ARTICLE
Journal of Cellular Biochemistry 112:916–923 (2011)

Journal of Cellular Biochemistry

Icariin is More Potent Than Genistein in Promoting Osteoblast Differentiation and Mineralization In vitro

Hui-Ping Ma,[1] Lei-Guo Ming,[2] Bao-Feng Ge,[2] Yuan-Kun Zhai,[2] Peng Song,[2] Cory J. Xian,[3] and Ke-Ming Chen[2*]

[1] *Department of Pharmacy, Lanzhou General Hospital, Lanzhou Command of CPLA, Lanzhou 730050, People's Republic of China*

[2] *Institute of Orthopaedics, Lanzhou General Hospital, Lanzhou Command of CPLA, Lanzhou 730050, People's Republic of China*

[3] *Sansom Institute for Health Research, University of South Australia, Adelaide, SA 5001, Australia*

ABSTRACT

There has been a strong interest in searching for natural therapies for osteoporosis. Genistein, an isoflavone abundant in soy, and icariin, a prenylated flavonol glycoside isolated from Epimedium Herb, have both been identified to exert beneficial effects in preventing postmenopausal bone loss. However, the relative potency in osteogenesis between the individual phytoestrogen flavonoids remains unknown. The present study compared ability of genistein and icariin in enhancing differentiation and mineralization of cultured rat calvarial osteoblasts in vitro. Dose-dependent studies in osteoblast differentiation measuring alkaline phosphatase (ALP) activity revealed optimal concentrations of genistein and icarrin for stimulating osteogenesis to be both at 10^{-5} M. Time course studies comparing the two compounds both at 10^{-5} M demonstrated that icariin treatment always produced higher ALP activity, more and larger areas of CFU-F_{ALP} colonies and mineralized nodules, more osteocalcin secretion, and calcium deposition, and a higher level of mRNA expression of osteogenesis-related genes COL1α2, BMP-2, OSX, and RUNX-2. However, they inhibited the proliferation of osteoblasts to a similar degree. In conclusion, although future in vivo studies are required to investigate whether icariin is more efficient in improving bone mass and/or preventing bone loss, our in vitro studies have demonstrated that icariin has a stronger osteogenic activity than genistein. In addition, while the prenyl group on C-8 of icariin could be the active group that takes part in osteoblastic differentiation and explains its greater potency in osteogenesis, mechanisms of action, and reasons for the relative potency of icariin versus genistein need to be further studied. J. Cell. Biochem. 112: 916–923, 2011.

KEY WORDS: GENISTEIN; ICARIIN; OSTEOBLASTS; DIFFERENTIATION; MINERALIZATION; OSTEOPOROSIS

Osteoporosis has become a worldwide significant clinical problem that is expected to worsen with aging populations around the world. Since the Women's Health Initiative has reported that the health risks of hormone replacement therapy (HRT) exceed benefits [The writing group for the women's Health Initiative investigators, 2002], more people have turned or are turning to alternative or natural therapies for osteoporosis treatment [Borrelli and Ernst, 2010]. Of all the natural alternatives currently under investigation, genistein, an isoflavone abundant in soy, has received most attention. Numerous studies using cultured bone cells, ovariectomized rat models and clinical trials support the conclusion that genistein might provide an alternative to prevent postmenopausal bone loss [Setchell and Lydeking-Olsen, 2003; Ullmann et al., 2005; Atmaca et al., 2008]. In traditional Chinese Medicine, osteoporosis is considered to be a disorder caused by the insufficiency of *kidney yang*, and the herbs perceived to be able to tonify the *kidney yang* have been used for more than 1000 years and today are still being used for osteoporosis treatment. Among the kidney-tonifying herbs, Epimedium Herb is one of the most frequently prescribed and has been extensively studied by modern

Hui-Ping Ma, Lei-Guo Ming-These two Authors Contributed Equally

Grant sponsor: Gansu Provincial Science & Technology Department, P.R. China; Grant number: 092NKDA025.

*Correspondence to: Ke-Ming Chen, Institute of Orthopaedics, Lanzhou General Hospital, Lanzhou Command of CPLA, Lanzhou 730050, P.R. China. E-mail: chkeming@yahoo.com.cn

Received 20 December 2010; Accepted 20 December 2010 • DOI 10.1002/jcb.23007 • © 2010 Wiley-Liss, Inc.
Published online 29 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

researchers. Icariin, a prenylated flavonol glycoside, has been isolated from the herb and found to be one of the main effective constituents [Guo et al., 1996].

Recently, we and others have demonstrated that icariin enhances osteogenic differentiation of rat bone marrow stromal cells, improves maturation and mineralization of osteoblasts in vitro, and suppresses osteoclastogenesis and inhibits bone resorption activity in vivo [Chen et al., 2005, 2007; Huang et al., 2007; Hsieh et al., 2010a,b]. Experiments in rats indicated that icariin prevented ovariectomy (OVX)-induced bone loss and reduction in femoral strength [Nan et al., 2009; Mok et al., 2010]. In addition, a 24-month randomized double-blind placebo-controlled clinical trial showed that Epimedium-derived phytoestrogen flavonoids (a daily dose of 60 mg icariin, 15 mg daidzein, and 3 mg genistein) were able to exert beneficial effects in preventing bone loss in postmenopausal women, without resulting in a detectable hyperplasia effect on the endometrium [Zhang et al., 2007].

However, the relative potency in anti-osteoporosis application among the individual phytoestrogen flavonoids remains to be investigated. As a step to address this question, the ability of the two main compounds (icariin and genistein) in enhancing the differentiation and mineralization of osteoblasts was compared in vitro. In addition, comparison of the osteogenic potency between two compounds with some molecular structural differences may give us clues about the structure–activity relationships of these osteotrophic phytoestrogen flavonoids.

MATERIAL AND METHODS

REAGENTS

Icariin (purity > 99%) and genistein (purity > 99%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Bejing, China). Minimal essential medium (MEM) was obtained from Invitrogen (Auckland, Scotland, UK). Fetal bovine serum (FBS) was the product of Lanzhou National Hycolone Bio-Engineering Co (Lanzhou, China). Collagenase II and trypsin were purchased from Gibco BRL (Gaithersburg, MD, USA). MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide), β-glycerophosphate, dexamethasone, ASAP (ascorbic acid 2-phosphate) were all from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

ANIMALS

Newborn Wistar rats were obtained from the Experimental Animal Center, Lanzhou University. Animal care and experiments were approved and conducted in accordance with accepted standards of animal care and use as deemed appropriate by the Animal Care and Use Committee of Lanzhou University, Lanzhou, China.

ISOLATION AND CULTURE OF RAT CALVARIAL OSTEOBLASTS

Calvarias were dissected aseptically from 10 newborn Wistar rats. Frontal and parietal bones were cleaned of adhering soft tissues and cut into about 1-mm^3 pieces. They were digested at 37°C for 20 min with shaking with an enzymatic solution containing 1 mg/ml collagenase II and 0.5 mg/ml trypsin in MEM. This procedure was repeated for three times, and the pieces were then further digested with 1 mg/ml collagenase II twice each for 60 min. The two supernatants were pooled and centrifuged at 1000 rpm for 5 min, and the cells were suspended in MEM containing 10% FBS and plated in 100-mm tissue culture dishes (Nunc, Roskilde, Danmark) supplemented 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 with humidification. The culture medium was changed every 3–4 days. After reaching confluence, the cells were detached by treatment with 1 mM EDTA and 0.25% trypsin and subcultured as described below for differentiation or proliferation assay.

DETERMINATION OF THE OPTIMAL CONCENTRATION FOR IMPROVING OSTEOGENIC DIFFERENTIATION

The optimal concentration of icariin or genistein for stimulating osteogenesis was determined using an osteoblast differentiation assay by comparing the activity of early bone formation marker alkaline phosphatase (ALP) in the culture. The cells were subcultured in 96-well tissue culture plate (Nunc, Danmark) at 3000 cells/well. When cells reached confluence (Day 0), the medium containing 10^{-8} M dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ASAP was added to induce osteogenic differentiation. Icariin or genistein was supplemented at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M, respectively, (n = 6 wells for each concentration). Equal volume of vehicle (1 μl DMSO/ml medium) was added in the control. After culture for 6 days, the ALP activity was determined biochemically with a modified method of King [Powell and Smith, 1954] using a commercial kit as instructed (Nanjing Jiancheng Bioengineering Ltd, Nanjing, China). The optimal concentration producing the highest ALP activity (absorbance measurement of the reaction product) can be determined by comparing the absorbance readings of cultures with different icariin or genistein concentrations.

TIME COURSE MEASUREMENT OF ALP ACTIVITY AND HISTOCHEMICAL STAINING

Cells were subcultured in 60-mm dishes (Nunc) at 5000 cells/cm^2. When cells reached confluence, the osteogenic medium was added, with icariin or genistein supplementation at the optimal concentration as determined above. The cellular ALP activity of the culture was measured on day 3, 6, 9, and 12, respectively (n = 3 per time point). The cells were rinsed twice and sonicated in 2 ml of 50 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100 and 2 mM $MgCl_2$ for 15 s, and the ALP activity in the sonicate was measured as above. Protein concentrations were determined with a BCA protein assay kit (Thermo, Rockford, USA). The ALP data are expressed as nmol phenol/15 min/mg protein. To further compare the potency of icariin and genistein in stimulating osteogenic differentiation, the numbers of colonies positive for ALP (CFU-F_{ALP}) were compared on day 9. The cells were fixed in 3.7% formaldehyde and 90% ethanol solution for 5 min, washed and then stained for 15–20 min at 37°C in 20 ml Michalis buffer, pH 8.9, containing 10 mg 1-naphthyl phosphate sodium and 10 mg fast blue RR salt. The numbers and total areas of blue colonies were measured by an Olympus SP 1000 Image Analyzer.

OSTEOCALCIN SECRETION AND MINERALIZATION ASSAYS

To examine effects of icariin or genistein on osteoblast functions in producing osteocalcin and mineralization, osteocalcin levels in cultures were measured and formation of mineralized nodules were determined. Levels of osteocalcin (produced by osteoblasts and secreted into culture medium) in 0.5 ml of the media collected during days 0–3, days 3–6, days 6–9, and days 9–12, respectively, were measured (ng/ml) with a Rat-Mid™ Osteocalcin EIA kit (Immunodiagnostic Systems Ltd, Fountain Hills, USA). Calcium deposition measurements were performed on days 3, 6, 9, and 12, respectively. Briefly, the cultures were rinsed twice with PBS and decalcified for 24 h by 2 ml of 1 N HCl, and calcium contents in the HCl supernatant samples were measured with a calcium colorimetric assay kit (BioVision, Mountain View, CA). The results were expressed as mg/dish. Histochemical alizarin red staining of mineralized nodules was carried out on day 12. Briefly, the cells were fixed in 3.7% formaldehyde for 10 min and stained by 0.1% alizarin red for 1 h at 37°C. The numbers and total areas of red nodules were measured by the Olympus SP 1000 Image Analyzer.

REAL-TIME PCR QUANTIFICATION OF GENE EXPRESSION

Effects of icariin and genistein in stimulating osteogenic gene expression were also examined by quantitative RT-PCR. The mRNA expression of bone protein collagen-I (COL1), osteogenic growth factor BMP-2, and osteogenic transcription factors OSX and Runx-2 was quantitatively determined after 0, 6, 12, 48, and 72 h of osteogenic induction and with and without supplement of icariin and genistein. Total RNA was extracted from the cells using RNAiso Kit (Takara Biotechnology, Dalian, China). Single-stranded cDNA was synthesized from 1 μg total RNA with a Primescrip™ RT reagent kit (Takara Biotechnology). Real-time PCR was performed using 2 μl of cDNA product in a 25-μl reaction volume with 7300 Real Time PCR System (Applied Biosystems, Singapore). SYBR® Premix Ex Taq™ II (Takara Biotechnology), specific primers (see below), and 2 μl of cDNA were used in each PCR reaction (95°C for 30 s, 40 cycles of denaturation at 94°C for 5 s, and annealing and extension at 60°C for 30 s). The sense and antisense primers were designed with the Primer Express 3.0 based on published rat cDNA sequences. GAPDH was used as an internal control gene. Primer sequences were as follows: COL1α2, 5′-TTCCCGGTGAATTCGGTCTC-3′ and 5′- ACCTCGGATTCCAATAGGACCAG-3; BMP-2, 5′-ACCGTGCTCAGCTTCCATCAC-3′ and 5′-TTCCTGCATTTGTTCCCGAAA-3′; OSX, 5′-GCCTACTTACCCGTCTGACTTT-3′ and 5′-GCCCACTATTGCCAACTGC-3′; Runx-2, 5′-GCACCCAGCCCATAATAGA-3′ and 5′-TTGGAGCAAGGAGAACCC-3′; GAPDH, 5′-GGCACAGTCAAGGCTGAGAATG-3′ and 5′-ATGGTGGTGAAGACGCC AGTA-3′. All real-time PCR reactions were performed in triplicate, and the results after calibration with GAPDH expression were calculated using the $^{\Delta\Delta}$CT method and are presented as fold increase relative to the non-stimulated control (at 0 h time point).

CELL PROLIFERATION ASSAY

Effects of icariin or genistein on osteoblastic proliferation were also determined. The isolated cells were subcultured in 96-well tissue culture plates (Corning) at 3000 cells/well and allowed to grow for 24 h. Icariin or genistein was added in the culture medium at their respective optimal concentration (n = 6 wells for each compound). Equivalent volume of vehicle in basal medium was used as the non-stimulated control. Cell proliferation was assessed by MTT assay after 48 and 72 h, respectively [Tumber et al., 2000]. Briefly, 10 μl of 5 mg/ml MTT solution was added to each well and incubated for 2 h, and then 100 μl of DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The plates were read on a microplate reader at a wavelength of 570 nm.

STATISTICAL ANALYSIS

Statistical analyses were carried out with the SPSS 16.0 software. Data for MTT assay were from six parallel experiments and others from triplicate experiments. All data shown are means ± SD. The significance levels were determined by ANOVA. Multiple comparisons were carried using the Tukey method. A value of $P < 0.05$ was taken as a significant difference.

RESULTS

To compare the function of genistein and icariin in osteogenic differentiation of osteoblasts, their dose-dependent effects and respective optimal concentrations need to be determined firstly. A high-performance assay method was applied in which ALP activity was used as an indicator. Dose-dependent studies revealed that the optimal concentrations of both genistein and icariin were at 10^{-5} M (Fig. 1A,B).

The ability of icariin and genistein both at 10^{-5} M in stimulating ALP activity was then compared in a time course (Fig. 1C). At all time points analyzed, icariin treatment always produced a higher ALP activity than the control group ($P < 0.01$) and than the genistein group ($P < 0.01$). On the other hand, ALP activity induced by the genistein group was not significantly higher than the control on day 3 and was lower than those of the icariin group at all the time points analyzed.

The histochemical staining for ALP on day 9 (Fig. 2A) revealed that the icariin group (I group) produced significantly more CFU-F_{ALP} colonies of larger areas than the genistein group (G group), and that the G group produced only slightly more and larger CFU-F_{ALP} colonies than the control group ($P > 0.05$) (Fig. 2B,C).

Osteocalcin is a non-collagen protein produced by osteoblasts and a marker for bone formation. During the early stage of osteogenic culture (Days 0–3), there were no differences among three groups in the osteocalcin concentration ($P > 0.05$). In the following days, with the increase of osteocalcin secretion, the differences between treatment groups became more obvious (Fig. 3A). Osteocalcin levels of the genistein-treated group were nearly two times higher than those of the control group during days 3–6 and days 6–9, but significantly lower than those of icariin group. During days 9–12, osteocalcin secretion declined and the differences between genistein group and the control disappeared; and yet the icariin group still produced more osteocalcin than the other two groups ($P < 0.05$).

The calcium deposition level is a direct indicator of mineralization. There were no significant differences among three groups on day 3. Beginning from day 6, the calcium contents increased

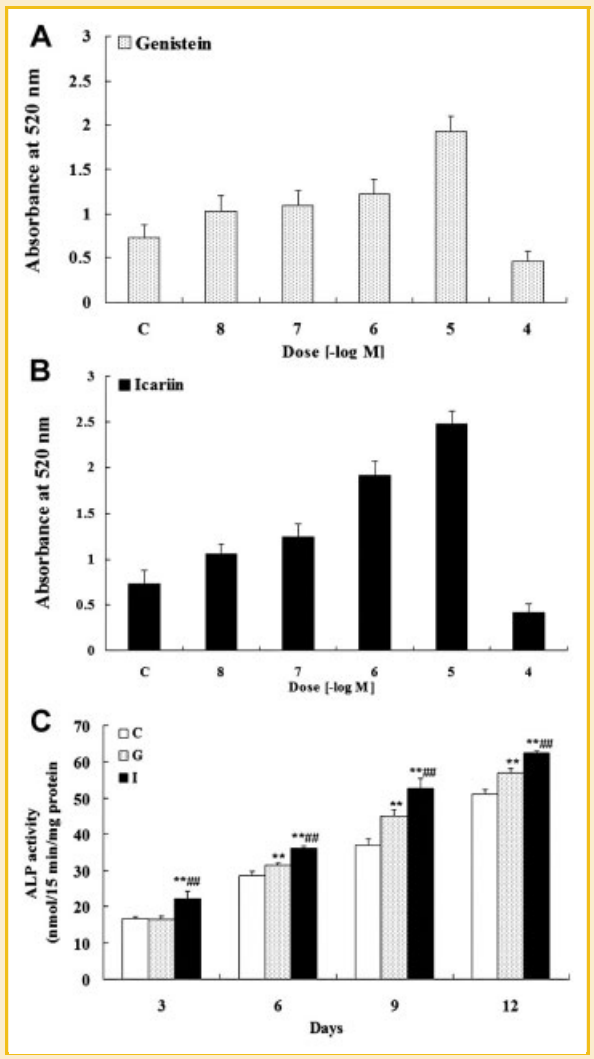

Fig. 1. Dose-dependent effects of genistein (A) and icariin (B) in osteogenic differentiation of neonatal rat calvarial osteoblasts (ROB) grown in 96-well culture plates and treated for 6 days with various concentrations of genistein and icariin, with ALP activity being measured (the absorbance at 570 nm, means ± SD of six replicate cultures, $^{**}P < 0.01$). C: Time course induction of ALP activity during 12 days of osteogenic culture, in which genistein (G) or icraiin (I) was supplemented at 10^{-5} M (means ± SD of triplicate cultures). $^{**}P < 0.01$ versus control, $\#\#P < 0.01$ versus G group.

steadily. Starting from day 6, the I group was always higher than the G group ($P < 0.01$), and was two times higher than the control (Fig. 3B).

The mineralized nodule formation assay on day 12 revealed a similar tendency as with calcium deposition (Fig. 4A). There were far more mineralized nodules of larger areas in the I group than in the G group, and mineralized nodules in the G group were significantly more and larger than those in the control group (Fig. 4B,C).

Type-I collagen is the most abundant protein in bone that is produced by osteoblasts. In the current study, active expression of type-I collagen COL1α2 gene suggests active bone matrix synthesis in cultured osteoblasts. Real-time PCR analysis revealed that COL1α2 mRNA expression increased at 6 h, peaked at 12 h after culture, and then declined gradually (Fig. 5A). Consistent with data from osteoblast differentiation and osteogenesis assays, the I group always induced the highest COL1α2 expression which was 2–3 times higher than the control. The G group produced significantly higher levels of COL1α2 than the control from 6 to 48 h ($P < 0.05$). After 72 h, no significant differences were found between G group and the control.

The stimulating effect of I group on mRNA expression of osteogenic growth factor BMP-2 was considerable (Fig. 5B). While the G group induced the highest level after 12 h, which was about 5 times higher than the control and 2 times higher than I group, the I group produced a peak BMP-2 expression after 24 h which was 30 times higher than the control and 10 times higher than G group. In addition, while the I group always produced higher levels of BMP-2 than the control from 6 to 72 h, G group produced more BMP-2 than the control only from 6 to 24 h.

Runx-2 and Osterix (OSX) are two critical transcription factors for osteoblast differentiation and bone formation [Nakashima et al., 2002]. The expression levels of Runx-2 mRNA increased and decreased synchronically in all three groups, which all peaked at 48 h (Fig. 5C). However, the I group produced the highest levels of Runx-2 at all time points, with the differences of the I group with G group and with the control being statistically significant. The G group expressed higher levels of Runx-2 than the control from 6 to 48 h, but lower than the control after 72 h ($P < 0.05$).

The OSX mRNA expression was affected by a different pattern (Fig. 5D). While the OSX expression peaked at 48 h among the time points examined in the control group, the G group and I group got to the peak expression levels 24 h earlier than the control. In addition, the I group produced 16 times more OSX than the control and 10 times more than the G group at the peak expression time point. When the control got to the peak level at 48 h, it was 3 times higher than the I group, and the I group was the lowest. After 72 h, the control was still higher than I group ($P < 0.05$), and G group was the lowest.

The effect of the two compounds on osteoblastic cell proliferation was also investigated. As shown in Figure 6, proliferation (as expressed by the absorbance value at 570 nm) in the I group and the G group was significantly lower than that of the control after 48 and 72 h ($P < 0.05$), indicating that icariin and genistein inhibited the proliferation of osteoblastic cells. No significant differences were found between the I group and G group.

DISCUSSION

Recently, there has been strong interest in studying the efficacy of phytoestrogen flavonoids as a potential alternative therapy for improving bone formation and preventing or treating osteoporosis. The current study has compared the relative ability of two phytoestrogen compounds (icariin and genistein) in enhancing the differentiation and mineralization of osteoblasts in vitro. Here, icariin was found to have a stronger ability than genistein in improving osteoblast differentiation and osteogenic function as demonstrated by ALP activity, osteocalcin secretion, calcium

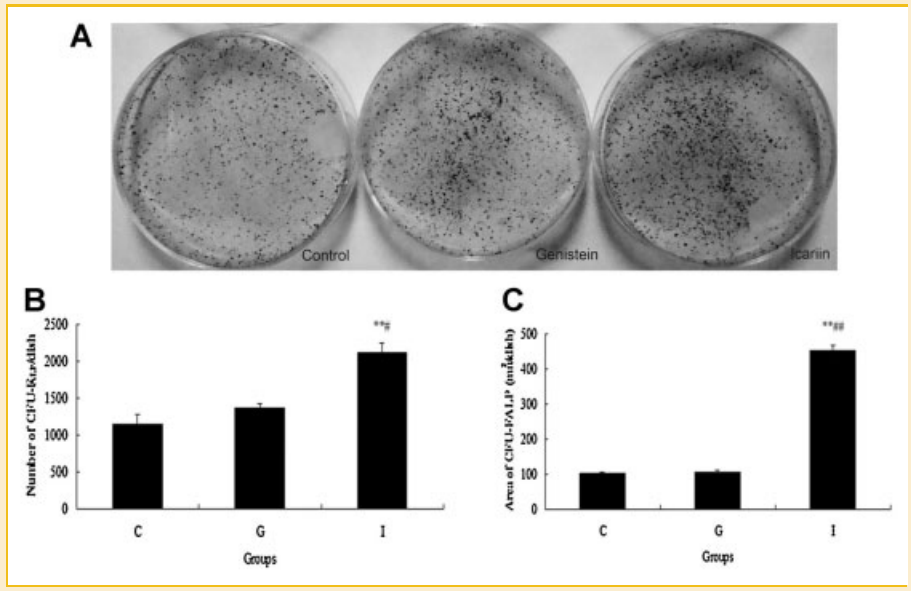

Fig. 2. ALP$^+$ stained CFU-F colonies formed by ROBs after 9 days of osteogenic induction culture (A), in which genistein or icariin was supplemented at 10^{-5} M. The number (B) and total area (C) of CFU-F_{ALP} colonies were measured (means ± SD of triplicate cultures). $^{**}P < 0.01$ versus control (C), $\#\#P < 0.01$ versus G group.

deposition and the number and area of mineralized bone nodules as well as stimulating the gene expression of COL1α2, BMP-2, OSX, and Runx-2. In addition, it was found that icariin inhibited the proliferation of osteoblasts in vitro to a similar degree as with genistein.

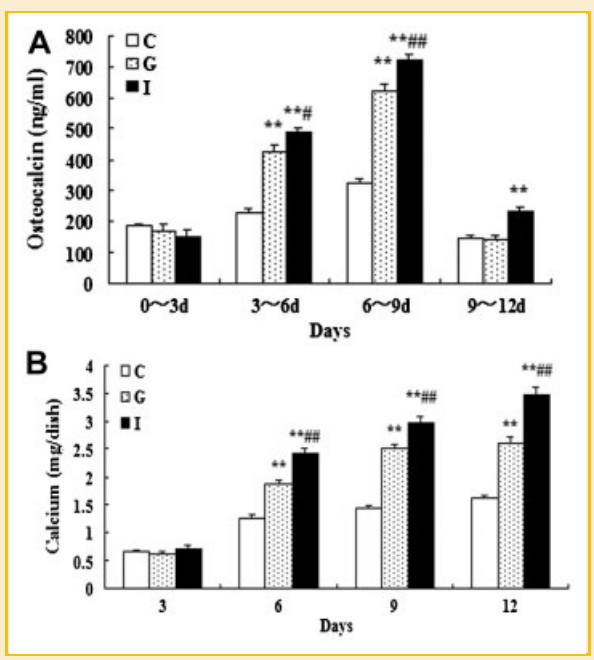

Fig. 3. Time course changes in osteocalcin concentration (A) in the conditioned osteogenic culture medium and calcium deposition (B) of cultured ROBs. Genistein (G) or icraiin (I) was supplemented at 10^{-5} M. Results are the means ± SD of triplicate cultures. $^{**}P < 0.01$ versus control (C); $\#P < 0.05$, $\#\#P < 0.01$ versus G group.

It is known that Osterix expression is downstream to Runx2 and its time course expression seems to follow that of Runx2 closely [Nakashima et al., 2002]. In the current study, it was observed that in the control group, expression levels of Runx2 and OSX both peaked at 48 h among the time points examined. After genistein or icariin treatment, however, while the peak level of Runx2 was still at 48 h, that of OSX occurred at 24 h. This suggests that genistein or icariin treatment can possibly speed up osteoblast differentiation or maturation by promoting earlier expression of OSX. Further studies are required to investigate this phenomenon.

Genistein has been extensively studied as one of the main phytoestrogens. Due to its structure similarity to 17β-estradiol (Fig. 7A), genistein can compete with estrogen for estrogen receptors (ER). The bone-conserving action is considered to result from the direct ER-mediated action of genistein on osteoblasts and their precursor cells [Chang et al., 2003; Chen et al., 2003; Rickard et al., 2003; Morris et al., 2006], and the inhibition of bone resorption maybe the indirect actions of genistein on osteoclasts by its inhibitory effects on levels of expression of osteoclastogenic and proinflammatory cytokines produced by osteoblasts [Viereck et al., 2002]. Apart from its affinity to ERs, genistein enhances osteogenic differentiation of bone marrow mesenchymal cells partly via nitric oxide/cGMP pathway or p38 MAPK-Runx-2 pathway [Pan et al., 2005; Okumura et al., 2006; Liao et al., 2008]. In addition, genistein represses adipogenic differentiation of human marrow stromal cells, which may contribute to the maintenance of human bone homeostasis [Heim et al., 2003].

The bone-preserving or osteotrophic action of icariin has also been investigated in recent years. However, the knowledge about its action mechanism is up to now still limited. It has been reported that icariin induces osteogenic differentiation in a BMP-2, SMAD4, Runx-2, or ER dependent manner [Hsieh et al., 2010a; Zhao et al., 2008; Mok et al., 2010], and it inhibits osteoclast differentiation and

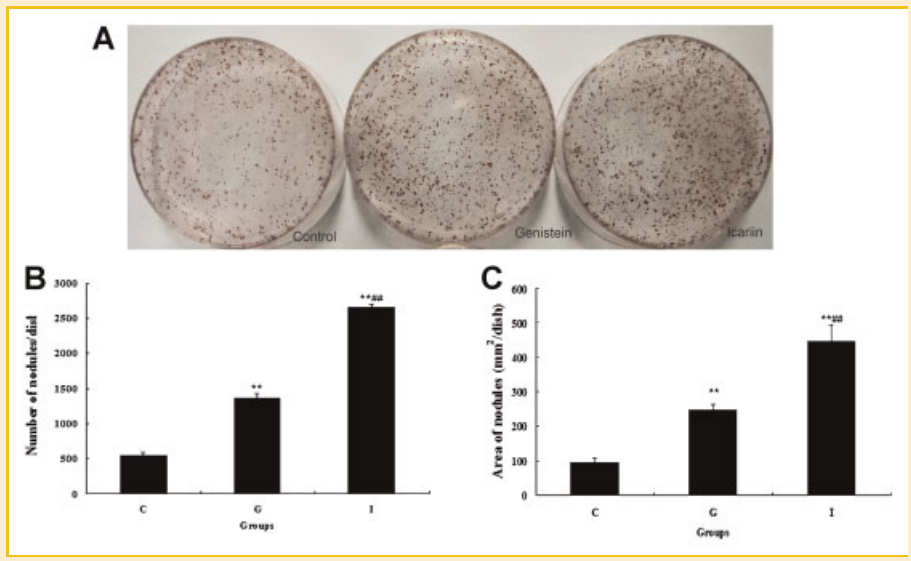

Fig. 4. Mineralized nodules formed by ROBs after 12 days of osteogenic induction culture as demonstrated by alizarin red staining (**A**). Genistein (G) or icariin (I) was supplemented at 10^{-5} M from the beginning of induction. Numbers (**B**) and total areas (**C**) of mineralized nodules were measured (means ± SD of triplicate cultures). $^{**}P < 0.01$ versus control, $\#\#P < 0.01$ versus G group.

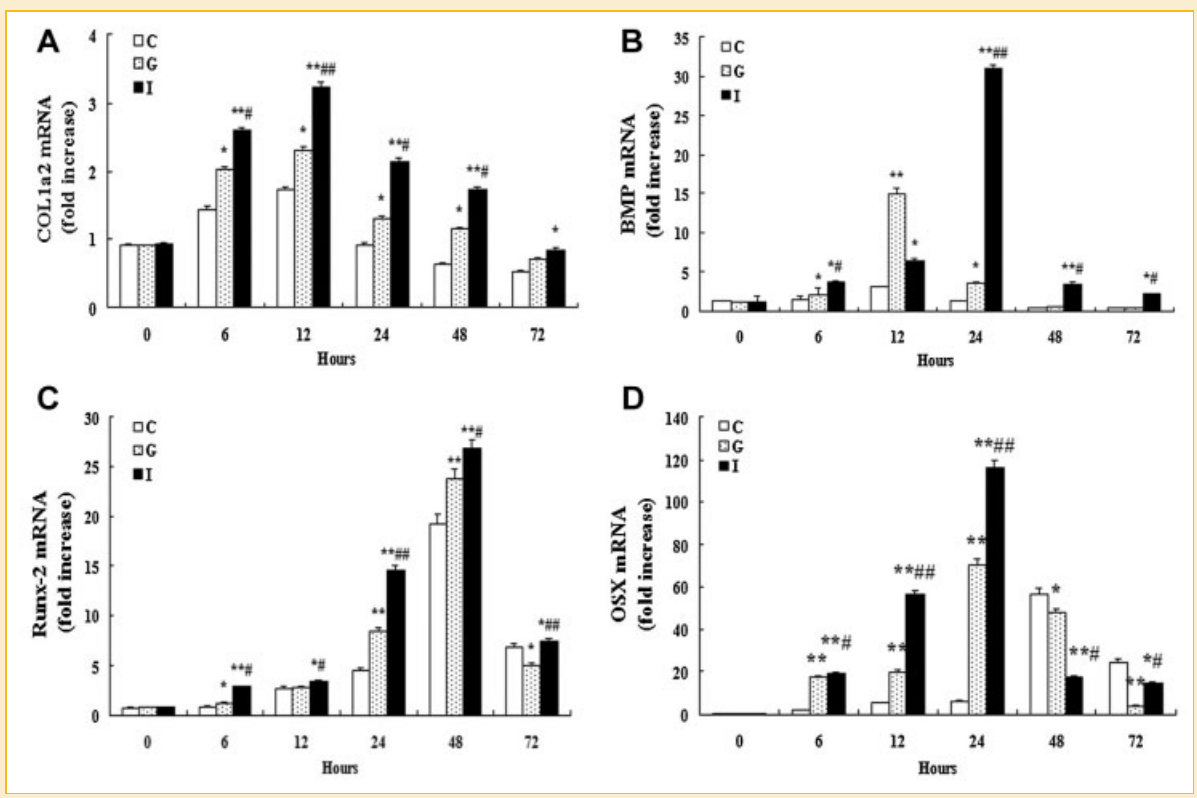

Fig. 5. Time course changes in mRNA expression of (**A**) COL1α2, (**B**) BMP-2, (**C**) Runx-2, and (**D**) OSX in ROBs during 72 h of osteogenic induction culture. Genistein (G) or icariin (I) was supplemented at 10^{-5} M. Results are expressed as the mean ± SD fold increase relative to the non-supplemented (time 0 h culture) control (n = 3 culture assays). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $\#P < 0.05$, $\#\#P < 0.01$ versus G group.

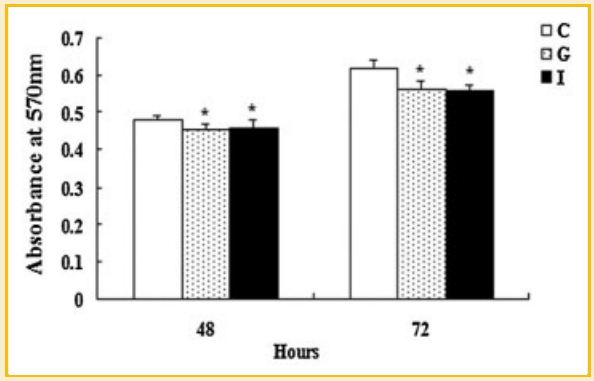

Fig. 6. Effects of icariin or genistein on proliferation of ROBs (MTT proliferation assay after 48 and 72 h culture). Icariin or genistein was supplemented into the culture medium of osteoblasts, respectively, at 10^{-5} M. Results are the absorbance values at 570 nm (mean ± SD, n = 6 assays).* $P < 0.05$ versus control.

bone resorption by suppressing activation of MAPKs/NF-kappaB [Hsieh et al., 2010b]. In addition, icariin is a protaglandin-E5 inhibitor [Ning et al., 2006; Dell et al., 2008] and enhances the production of bioactive nitric oxide [Xu and Huang, 2007], as well as mimicking the effects of testosterone [Zhang and Yang, 2006].

In the present study, icariin was found to have a stronger ability than genistein in improving osteoblast differentiation, mineralization, and expression of osteogenesis-related genes, suggesting that icariin may exert better effects than genistein in preserving bone mass or preventing bone loss. Icariin is more complex in chemical structure compared to genistein. It is a prenylflavonoid glycoside with a glucosyl group on C-3 and a rhamnosyl group on C-7 as well as a methoxyl group on C-4 (Fig. 7B). Two derivatives, icaritin, and desmethylicaritin, have been made, which are in non-conjugated form and hydroxylated at C-3, C-7, and C-3, C-7, C-4 positions, respectively. It was reported that icaritin and desmethylicaritin are more potent in enhancing osteoblastic differentiation [Wang and Lou, 2004; Ye and Lou, 2005; Huang et al., 2007], which suggested that the glucosyl group, rhamnosyl group, and methoxyl group are not necessary for osteogenic activity. Several studies demonstrated that the prenyl group on C-8 may be highly related to the osteogenic activity. Zhang et al. [2008] and Kretzschmar et al. [2010] compared genistein derivatives including 6-prenylgenistein, 8-prenylgenistein, and 6, 8-prenylgenistein in their effects on osteoblastic proliferation, differentiation, and mineralization in UMR 106 cells, and found that prenylation at C-8, but not C-6, could increase the bone-protective effect of genistein. These studies suggest that the prenyl group on C-8 of icariin is an active group that takes part in osteoblastic differentiation, and this may explain why icariin is more potent than genistein in stimulating osteogenesis.

The significance for our finding that icariin is more potent than genistein in promoting bone formation in vitro is of 3-folds. Firstly, it provides scientific evidence and support for the belief in traditional Chinese medicine that Epimedium herb, from which icariin was isolated, has the ability to strengthen bones and has long been used to treat osteoporosis. Secondly, genistein has been extensively studied by animal experiments and clinical trials, but it's still not approved for clinical application because of its controversial or inconsistent results. A compound with obvious higher activity in bone cells may settle this predicament. Our work indicated that icariin may be a better candidate than genistein for promoting bone health. Thirdly, the higher osteogenic activity of icariin than genistein could be endowed by the superiority of chemical structure of icariin (with a prenyl group on C-8 position). As a prenylated flavonol glycoside, icariin may represent a class of compounds with higher osteogenic activity than conventional phytoestrogen, which has not been recognized and reported previously.

In conclusion, our in vitro studies have demonstrated that icariin has a stronger osteogenic activity than genistein. However, whether it's more efficient in preserving bone mass and preventing bone loss induced by oestrogen deficiency needs to be investigated in animal experiments. In addition, the mechanisms of action and the reason for the relative potency of icariin versus genistein in osteogenesis need to be further studied.

ACKNOWLEDGMENTS

This project was supported by grant 092NKDA025 from Gansu provincial Science & Technology Department, P.R. China. CJX is a senior researcher fellow of NHMRC Australia.

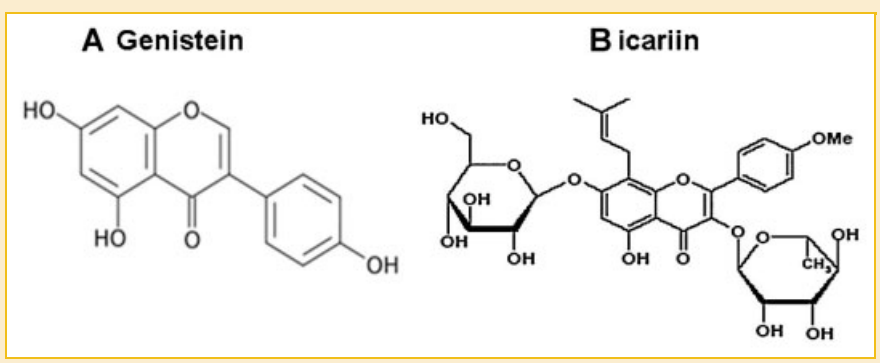

Fig. 7. Chemical structures of genistein (A) and icariin (B).

REFERENCES

Atmaca A, Kleerekoper M, Bayraktar M, Kucuk O. 2008. Soy isoflavones in the management of postmenopausal osteoporosis. Menopause 15:748–757.

Borrelli F, Ernst E. 2010. Alternative and complementary therapies for the menopause. Maturitas 66:333–343.

Chang H, Jin TY, Jin WF, Gu SZ, Zhou YF. 2003. Modulation of isoflavones on bone-nodule formation in rat calvaria osteoblasts in vivo. Biomed Environ Sci 16:83–89.

Chen X, Garner SC, Quarles LD, Anderson JJ. 2003. Effects of genistein on expression of bone markers during MC3T3-E1 osteoblastic cell differentiation. J Nutr Biochem 14:342–349.

Chen KM, Ge BF, Ma HP, Liu XY, Bai MH, Wang Y. 2005. Icariin, a flavonoid from the herb Epimedium enhances the osteogenic differentiation of rat primary bone marrow stromal cells. Pharmazie 60:939–942.

Chen KM, Ge BF, Liu XY, Ma HP, Lu MB, Bai MH, Wang Y. 2007. Icariin inhibits the osteoclast formation induced by RANKL and macrophage-colony stimulating factor in mouse bone marrow culture. Pharmazie 62:388–391.

Dell M, Galli GV, Dal Cero E, Belluti F, Matera R, Zironi E, Pagliuca G, Bosisio E. 2008. Potent inhibition of human phosphodiesterase-5 by icariin derivatives. J Nat Prod 71:1513–1517.

Guo BL, Wang CL, Xiao PG. 1996. Determination of flavonoids and quality evaluation of Sagittate Epimedium (Epimedium sagittatum). Chin Tradit Herb Drugs 27:524–526.

Heim M, Frank O, Kampmann G, Sochocky N, Pennimpede T, Fuchs P, Hunziker W, Weber P, Martin I, Bendik I. 2003. The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells. Endocrinology 145:848–859.

Hsieh TP, Sheu SY, Sun JS, Chen MH, Liu MH. 2010a. Icariin isolated from Epimedium pubescens regulates osteoblasts anabolism through BMP-2, SMAD4, and Cbfa1 expression. Phytomedicine 17:414–423.

Hsieh TP, Sheu SY, Sun JS, Chen MH. 2010b. Icariin inhibits osteoclast differentiation and bone resorption by suppression of MAPKs/NF-kappaB regulated HIF-1alpha and PGE(2) synthesis. Phytomedicine May 27 [Epub ahead of print].

Huang J, Yuan L, Wang X, Zhang TL, Wang K. 2007. Icaritin and its glycosides enhance osteoblastic, but suppress osteoclastic, differentiation and activity in vitro. Life Sci 81:832–840.

Kretzschmar G, Zierau O, Wober J, Tischer S, Metz P, Vollmer G. 2010. Prenylation has a compound specific effect on the estrogenicity of naringenin and genistein. J Steroid Biochem Mol Biol 118:1–6.

Liao Qc, Xiao ZS, Qin YF, Zhou HH. 2008. Genistein stimulates osteoblastic differentiation via p38 MAPK-cbα1 pathway in bone marrow culture. Acta Pharmacol Sin 29:176–180.

Mok SK, Chen WF, Lai WP, Leung PC, Wang XL, Yao XS, Wong MS. 2010. Icariin protects against bone loss induced by oestrogen deficiency and activates oestrogen receptor-dependent osteoblastic functions in UMR 106 cells. Br J Pharmacol 159:939–949.

Morris C, Thorpe J, Ambrosio L, Matteo S. 2006. The soybean isoflavone genistein induces differentiation of MG63 human osteosarcoma osteoblasts. J Nutr 136:1166–1170.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Nan H, Ma MH, Nan SS, Xu LL. 2009. Antiosteoporotic activity of icariin in ovariectomized rats. Phytomedicine 16:320–326.

Ning H, Xin ZC, Lin G, Banie L, Lue TF, Lin CS. 2006. Effects of icariin on phosphodiesterase-5 activity in vitro and cyclic guanosine monophosphate level in cavernous smooth muscle cells. Urology 68:1350–1354.

Okumura N, Yoshikawa T, Lida J, Nonomura A, Takakura Y. 2006. Bone formation-promoting effect of genistein on marrow mechymal cell culture. Biomed Mater Eng 16:23–32.

Pan W, Quarles LD, Song LH, Yu YH, Jiao C, Tang HB, Jiang CH, Deng HW, Li YJ, Zhou HH, Xiao ZS. 2005. Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. J Cell Biochem 94:307–316.

Powell ME, Smith MJH. 1954. The determination of serum acid and alkaline phosphatase activity with 4-aminoantipyrine (A.A.P). J Clin Path 7:245–248.

Rickard DJ, Monroe DG, Ruesink TJ, Khosla S, Ring BL, Spelsberg TC. 2003. Phytoestrogen genistein acts as an estrogen agonist on human osteblastic cells through estrogen receptors alpha and beta. J Cell Biochem 89:633–646.

Setchell KDR, Lydeking-Olsen E. 2003. Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. Am J Clin Nutr 78:593S–609S.

The writing group for the women's Health Initiative investigators. 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal woman: principle results from the Woman's Health Initiative randomized controlled trial. JAMA 288:321–333.

Tumber A, Meikle MC, Hill PA. 2000. Autocrine signals promote osteoblast survival in culture. J Endocrinol 167:383–390.

Ullmann U, Bendik I, Fluhmann B. 2005. Bonistein™ (synthetic genistein), a food component in development for a bone health nutraceutical. J Physiol Pharmacol 56:79S–95S.

Viereck V, Grundker C, Blaschke S, Siggelkow H, Emons G, Hofbauer LC. 2002. Phytoestrogen genistein stimulates the production of osteoprotegerin by human trabecular osteoblasts. J Cell Biochem 84:725–735.

Wang ZQ, Lou YJ. 2004. Proliferation-stimulating effects of icaritin and desmethylicaritin in MCF-7 cells. Eur J Pharmacol 504:147–153.

Xu HB, Huang ZQ. 2007. Icariin enhances endothelial nitric-oxide synthase expression on human endothelial cells in vitro. Vascul Pharmacol 47: 18–24.

Ye HY, Lou YJ. 2005. Estrogenic effects of two derivatives of icariin on human breast cancer MCF-7 cells. Phytomedicine 12:735–741.

Zhang ZB, Yang QT. 2006. The testosterone mimetic properties of icariin. Asian J Androl 8:601–605.

Zhang G, Qin L, Shi Y. 2007. Epimedium-derived phytoestrogen flavonoids exert beneficial effect on preventing bone loss in late postmenopausal women: a 24-month randomized, double-blind and placebo-controlled trial. J Bone Miner Res 22:1072–1079.

Zhang Y, Li X, Yao XS, Wong MS. 2008. Osteogenic activities of genistein derivatives were influenced by the presence of prenyl group at ring A. Arch Pharm Res 31:1534–1539.

Zhao J, Ohba S, Shinkai M, Chung UI, Naqamune T. 2008. Icariin induces osteogenic differentiation in vitro in a BMP- and Runx2-dependent manner. Biochem Biophys Res Commun 369:444–448.