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Icariin stimulates the osteogenic differentiation of rat bone marrow stromal cells via activating the PI3K-AKT-eNOS-NO-cGMP-PKG signal pathway

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ABSTRACT

Icariin, a prenylated flavonol glycoside isolated from Epimedii herba, has been found to be a potent stimulator of osteogenic differentiation and has potential application in preventing bone loss. However, the signaling pathway underlying its osteogenic effect remains unclear. We hypothesized that the osteogenic activity of icariin is related to the nitric oxide (NO) signal pathway and PI3K/AKT pathway in its upstream. Rat bone marrow stromal cells (rBMSCs) were cultured in osteogenic medium and treated with icariin or together with L-NAME, ODQ, PDE5, or/and LY294002 (the inhibitor of NOS, sGC, cGMP, and PI3K respectively), and effects were examined on the expression of signal messengers (NOS, NO, sGC, cGMP, PKG and PI3K) and the levels of osteogenic markers (alkaline phosphatase or ALP, osteocalcin and calcified nodules). It was found that icariin dose-dependently increased ALP activity, and treatment at the optimal concentration (10^{-5} M) increased NOS activity, iNOS and eNOS expression, NO production, sGC and cGMP contents and PKG expression besides the phosphorylation of AKT. The addition of L-NAME, ODQ and PDE5 significantly inhibited the icariin effects on above markers respectively. The addition of LY294002 decreased the p-AKT level, NOS activity, eNOS expression and NO production significantly, but had no significant effect on iNOS expression. The addition of any of the four inhibitors also abolished the osteogenic effect of icariin on rBMSCs as indicated by ALP activity, osteocalcin synthesis, calcium deposition and the number and areas of calcified nodules. These results suggest that the osteogenic effect of icariin involves the PI3K-AKT-eNOS-NO-cGMP-PKG signal pathway. Furthermore, dosage response

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studies showed that icariin at 10^{-6} M (a physiologically achievable concentration in vivo) also activated this signal pathway.

Key Words: Icariin; Bone marrow stromal cells; Osteogenic differentiation; Nitric oxide; Signal transduction

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Introduction

Bone disorders, especially osteoporosis, are becoming increasingly prevalent due to the demographic changes and longer life expectancy. In particular, the postmenopausal osteoporosis is the wide spread form of osteoporosis, affecting 1 in 2 women over the age of sixty. Although hormone replacement therapy (HRT) has been the therapeutic measure for prevention and treatment of postmenopausal osteoporosis, the Women's Health Initiative reported that the health risks of HRT exceed benefits [1]. Bisphosphonates have been used to treat osteoporosis [2]. However, the logistics and high costs of their administration and occasional toxicities including the widely publicized incidence of osteonecrosis of the jaw are of concern [3], and the cost effectiveness of their widespread or long-term use has been questioned. In addition, despite the availability of an armamentarium of agents, the optimal agent remains a challenge [4]. Therefore, more and more people have turned to or are searching for alternative or natural therapies for osteoporosis [5].

The herb of the genus Epimedium has long been used in Traditional Chinese Medicine to treat bone fractures and prevent osteoporosis, as it is believed that the herb can strengthen bones and tendons. Experimental studies showed that total flavonoids of genus Epimedium could significantly up-regulate osteogenic and down-regulate adipogenic differentiation of bone marrow stromal cells in ovariectomized rats [6,7]. A 24-month randomized, double-blind and

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placebo-controlled trial found that *Epimedium*-derived phytoestrogen flavonoids exerted beneficial effects of preventing bone loss in late postmenopausal women without any detectable effect of hyperplasia in the endometrium [8]. These previous studies revealed that the flavonoids contained in the herb are highly related to its therapeutic effects.

Icariin, a prenylated flavonol glycoside, is considered to be the major pharmacologically active component of Epimedii herba, and the content of icariin has been employed as the standard for quality control in *Epimedii herba* and its drug preparations [9]. We and others have previously reported that icariin enhances the osteogenic differentiation of bone marrow stromal cells and inhibits the osteoclast formation [10-12]. The more recent studies showed that after administration of icariin, de novo bone formation was stimulated and bone mass increased against ovariectomy or glucocorticoid-induced osteoporosis [13-17]

The signaling pathway underlying the osteogenic effect of icariin remains unclear. Ye et al reported that icaritin and desmethylicaritin, the two derivatives of icariin, stimulated the proliferation of MCF-7 cells, and these effects were completely blocked by estrogen receptor inhibitor ICI 182,780, suggesting that the cell-proliferative effect of icaritin and desmethylicaritin was mediated by estrogen receptor [18,19]. Zhao et al reported that icariin exerts its osteogenic effect through induction of Runx2 expression and activation of BMP signaling [20]. Similarly, Hsieh et al found that the osteogenic activity of icariin was associated with increased

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expression levels of BMP-2, Smad4, Runx2, and OPG as well as decreased expression of the RANKL [21]. While the osteogenic effect on mouse BMSCs of genistein, an isoflavone contained in soybean, has been found to be partly through activation of the nitric oxide (NO)/cGMP pathway [22], it is unknown whether the osteogenic property of icariin involves activation of the NO pathway. Nitric oxide (NO), a signaling molecule synthesized from L-arginine by nitric oxide synthase (NOS), has been found to be produced by bone cells and may restrain osteoclast-mediated bone resorption and stimulate osteoblast activity [23-25]. Since icariin has been found to exert its therapeutic effect on erectile dysfunction by increasing the expression of nitric oxide synthase (NOS) in the corpus cavernosum of castrated rats [26,27], and icariin increased the production of NO and endothelial NO synthase (eNOS) expression in human endothelial cells [28], we hypothesize that the osteogenic effect of icariin involves the NO-sGC-cGMP-PKG pathway, which begins with activation of NOS, the enzyme that synthesizes NO in cells. The increased NO levels activate the soluble guanylyl cyclase (sGC), thus increasing the levels of cyclic guanosine monophosphate (cGMP) which acts to activate protein kinase-G (PKG), a cGMP-dependent protein kinase [29-31].

Furthermore, it has been reported that icariin delays homocysteine-induced endothelial cellular senescence and confers beneficial effects on cardiovascular system by activation of PI3K/AKT-eNOS signaling pathway [32]. Serine/threonine protein kinase AKT, also known as protein kinase B, plays an important role in a

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number of cellular processes including metabolic responses, survival, proliferation, differentiation and growth [33-36]. AKT protein kinases are stimulated by a number of receptor tyrosine kinases and G protein-coupled receptors through phosphatidylinositol 3-kinase (PI3K) [37]. In addition, it has been reported that AKT can phosphorylate eNOS, one isoform of NOS [38]. Since AKT signaling has been reported to be involved in osteoblast proliferation and skeletal development [39-41], here we further hypothesize that the osteogenic effects of icariin is related to both AKT and NO signal pathway, and AKT is upstream of NO pathway described above. In the current study, we identified that icariin stimulates the osteogenic differentiation of rat bone marrow stromal cells (rBMSCs) via the activation of the PI3K-AKT-eNOS-NO-sGC-cGMP-PKG signal pathway.

Materials and methods

Animals

Four adult male Wistar rats weighing 120-170g were obtained from the Animal Breeding Center of Gansu College of Traditional Chinese Medicine (Lanzhou, China). Procedures for obtaining bone marrow samples (for isolating bone marrow stromal cells) from these rats were carried out according to the *Guide for the Care and Use of*

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Laboratory Animals, published by the US National Institutes of Health, and approved by Animal Ethics Committee of Lanzhou General Hospital (Gansu, China).

Reagents

Icariin (ICA, purity > 99%) was purchased from National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China) and dissolved in DMSO. Culture media (DMEM/F12) and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). Penicillin and streptomycin were obtained from Gibco BRL (Gaithersburg, MD, USA). Majority of drugs were purchased from Sigma (Steinheim, Germany), including dexamethasone, β-glycerophosphate, ascorbic acid phosphate and cGMP Enzyme Immunoassay Kit (Sigma-Aldrich, St. Louis, MO, USA). Alkaline phosphatase activity measurement Kit and NOS activity measurement Kit were purchased from Nanjing Jiancheng Company (Nanjing, China). Calcium colorimetric Assay Kit was obtained from Biovision (San Francisco, CA, USA) and enzyme immunoassay ELISA Kit for the quantitative determination of osteocalcin was purchased from Immunodiagnostic Systems Ltd (Boldon, UK).

Cell culture and treatments

The primary culture of rBMSCs was established as described previously [42]. Tibias and femurs were immediately removed from humanely sacrificed rats and

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dissected from the attached muscles and tissues with an aseptic technique. The ends of the bones were removed, and marrow plugs were flushed out by injection of DMEM/F12 medium, containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The plugs were dispersed by repeated pipetting and forcefully passed through a 19-gauge needle finally filtered through a 76 μm stainless steel cell strainer in order to obtain a single cell suspension. The suspension was adjusted to 1×10^7 cells/ml and seeded into 100-mm culture dishes (Nunc, Denmark) at 6 ml/dish. After 72h culture at 37°C in 5% humidified CO_2 atmosphere, the medium was removed and all dishes were washed three times with phosphate buffered saline (PBS). At 80% confluence, the cells were detached by treatment with 1mM EDTA and 0.25% trypsin and subcultured. The cell-surface antigen expression and purity of the subcultured cells were determined by flow cytometry. When most of the cultured cells were identified to be MSCs expressing the expected stem cell markers, they were used to determine the optimal concentration of icariin for improving osteogenic differentiation.

To determine the optimal concentration of icariin in enhancing osteogenesis, the rBMSCs grown to 80% confluence were cultured in the osteogenic medium (containing 10^{-8} M dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid phosphate), and icariin was supplemented at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M, respectively (n = 6 wells for each concentration). The activity of early bone formation marker alkaline phosphatase (ALP) of the cultures was

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measured after 9 days, a time point when peak activity is usually obtained. The optimal concentration of icariin was determined by comparing the ALP activities obtained with different concentrations of supplemented icariin.

For analyzing signaling pathways during osteogenic differentiation, the rBMSCs grown to 80% confluence were cultured in the osteogenic medium (containing 10^{-8} M dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid phosphate) with or without icariin, 100 μM L-NAME (an inhibitor of eNOS and iNOS), 20 μM ODQ (an inhibitor for soluble guanylate cyclase or sGC), 0.02 Unit/mL PDE5 (a cGMP inhibitor) or 50μM LY294002 (a PI3K inhibitor). The osteogenic culture media with or without these supplements were refreshed every three days. The expression and enzyme activities of eNOS and induced nitric oxide synthase (iNOS), the production of NO in culture media, the intracellular content of sGC, cGMP, PKG, and p-AKT were measured or analyzed at different time of culture. Treatment effects on osteogenic differentiation were also examined at different time points by analyzing markers including ALP activity, osteocalcin secretion, calcium deposition and numbers and areas of calcified nodules. Icariin was added at the above determined optimal concentration. The final concentration of DMSO (as the solvent of icariin) in culture was less than 0.05%, a concentration that did not interfere with the test system [44].

Identification of Surface Phenotypes of rBMSCs

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A rBMSCs suspension (1×10^6 cells/mL) was prepared and washed twice with PBS, then incubated in the dark at room temperature with primary antibodies against CD45 (PE-labeled), CD90 (PE-labeled) and CD44 (FITC-labeled) and corresponding isotype control (all antibodies were purchased from BD Biosciences, Franklin Lakes, NJ, USA). The stained cells were rinsed by cold PBS and immediately subjected to flow cytometric analysis to determine the cell surface marker expression and purity of rBMSCs.

Measurement of total NOS activity

At 12h after the commencement of osteogenic culture, cells were washed with PBS twice and the activity of total NOS (including iNOS and eNOS) was determined in the conditioned medium using the NOS Kit following the procedures recommended by the manufacturer.

Measurement of NO

NO production in the culture medium was measured as nitrite/nitrate [25], using Griess Reagent (Molecular Probes, Invitrogen, Eugene, OR, USA). In this assay, equal amounts of 1% sulphanylic acid and 0.1% N-(1-naphthyl) ethylene diamine were added to the samples and then incubated for 30 min at room temperature. The resulting absorbance was measured at 543nm. The amount of NO in each sample was

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determined using a standard curve generated with known concentrations of NO and expressed as nmol NO/mL culture medium.

cGMP assay

Determination of intracellular cGMP [45] was carried out by cGMP Enzyme Immunoassay Kit following the manufacturer's directions. The rBMSCs were lysed in 0.1 M hydrochloric acid, and 50 μl aliquots of the cell lysates were transferred into 96-well plates and subjected to the enzyme immunoassay. Optical absorbance was measured at 405 nm. cGMP levels were corrected by sample protein concentrations as determined by BCA assay (Biosynthesis Biotechnology, Beijing, China), and data are shown as pmol/well.

Real Time RT-PCR analysis

The cultured cells were harvested and lysed at 12h after icariin and specific inhibitor treatment. Total RNA was extracted with Trizol reagent (Invitrogen, Eugene, OR, USA) according to the manufacturer's instruction. To remove any DNA contamination, RNA samples were treated with DNase I (Invitrogen). Using the RNA samples, cDNAs were prepared using the PrimeScript^{TM} RT reagent Kit (TaKaRa, Dalian, China). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed for iNOS, eNOS, sGC, PKG and internal control GAPDH under standard

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enzyme and cycling conditions on a 7300 real-time PCR system using relative quantitation gene expression assays (Applied Biosystems, Singapore). All reactions were carried out in triplicate; the data are expressed as amplification signal ratios of target gene/GAPDH control. The primer sequences are given in Table 1.

Western blots analysis

The cultured cells were harvested and lysed at 24h after icariin and specific inhibitor treatment. Cells were homogenized in the homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 0.1% SDS) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma). The crude homogenate was centrifuged at 4°C for 15 min at 12,000 rpm. The supernatants were collected and then the total protein contents were quantified by BCA assay Kit. Then, 10μg of total protein from each sample was separated by SDS-PAGE (12% gel) and transferred to PVDF membranes. After incubation in blocking solution (2% non-fat milk) for 2h at room temperature, membranes were incubated overnight at 4℃ with primary antibodies at 1:1000 dilution [rabbit anti-iNOS, rabbit anti-PKG and rabbit anti p-AKT (Cell Signaling Technology, USA), mouse monoclonal to sGC and rabbit anti-eNOS (Abcam, Hong Kong)], and loading control antibody (mouse anti β-actin polyclonal antibody) (1:500; Zhongshan Gold Bridge, Beijing, China). After three washes with PBS-Tween-20,

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membranes were incubated with 1:3000 dilution of second antibody for 2h, and the immunoreactions signals were detected with the enhanced chemiluminescence reagent (Millipore Corp, USA) and exposed on X-ray film (Kodak, China). Relative intensities were scanned using Image-Pro plus 6.0. The data (densities of the product bands) were expressed as relative optical density unit after being standardised against β-actin band of each sample.

Measurement of alkaline phosphatase (ALP) activity

At day 9 after osteogenic induction culture, the cells were rinsed twice and sonicated in 2ml of 50 mM Tris-HCl, pH7.2, containing 0.1% Triton X-100 and 2mM $MgCl_2$ for 15 seconds, and the ALP activity in the sonicate was measured biochemically with a modified method of King (Powell and Smith, 1954) using a commercial kit as instructed (Nanjing Jiancheng Bioengineering Ltd, Nanjing, China) [43]. 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.

Determination of osteocalcin

The level of osteocalcin secreted into the culture medium at 9^{th} day was determined by enzyme immunoassay (ELISA) using a rat osteocalcin Kit

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(Immunodiagnostic Systems Ltd., Boldon, U.K.) following the manufacturer's directions [43]. The osteocalcin amount was expressed as μg/ml culture medium.

Calcium deposition level assay

The calcium deposition level was measured at 12th day of the osteognic induction culture. The cell layer was rinsed twice with PBS and incubated in 1 ml of 1 mol/L HCl overnight with gentle shaking. The released Ca^{2+} in the sample was quantified by the O-cresolphthalain complex method with Calcium Colorimetric Assay Kit (BioVision, K380-250). Results were expressed as μg/well [46].

Calcified nodules formation assay

Calcified nodule formation in rBMSCs cultures was determined by staining with Alizarin Red-S which binds selectively to calcium (Sigma). After 14 days of osteogenic induction culture, cells were washed twice with PBS (pH 7.4), fixed with 10% formaldehyde for 10min, washed three times with distilled water, and then incubated with 40 mmol/L Alizarin Red-S for 1h at 37°C. After staining, cultures were washed three times with deionized water. Stained cultures were photographed, and areas and relative intensities of stained nodules were scanned using Image-Pro plus 6.0 software.

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Statistical analysis

The data are presented as mean ± standard deviation (*SD*). Each treatment group had at least three replicates (n=3), and each experiment was repeated 3 times. All data were analyzed using one-way ANOVA followed by the LSD post hoc test using SPSS 16.0 (Chicago, IL, USA). Differences between means were considered statistically significant when *P*-values <0.05.

Results

Characterization of surface phenotypes of rBMSCs

Expression of MSC surface markers and purity of rBMSCs were examined. As shown in Fig. 1A, the cells at passage 2 were positive to CD44 (73.09%), CD90 (>94.94%) and negative to CD45 (4.93%). The flow cytometry data indicated that most of the cells expressed standard surface markers of MSCs and thus were used for experiments described below.

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ICA improves osteogenic differentiation in a dose-dependent manner

Prior to investigating the signaling pathways involved in icariin activity in promoting osteogenic differentiation, the concentration of icariin with the strongest osteogenic activity needs to be determined. As shown in Fig. 1B, the ALP activity was increased by icariin in a dose-dependent manner. It began to be significantly higher than that of control at 10^{-7} M (P<0.05), became much higher at 10^{-6} M (P<0.01), and peaked at 10^{-5} M, then decreased significantly at 10^{-4} M. Since the strongest activity was obtained by 10^{-5} M icariin, this concentration was used in the following experiments.

ICA increases NOS activity and NO production

To investigate whether the NO pathway is involved in the osteogenic effect of ICA, activity levels of NOS, an enzyme catalyzing the production of NO from L-arginine, were firstly assayed after 12h of osteogenic induction culture. The total NOS activity (including iNOS and eNOS) in the ICA-supplemented culture was significantly higher than that of the control group (P<0.01) (Fig. 2A). The NO content of the ICA group was also increased significantly (P<0.01) (Fig. 2B). Consistently, the mRNA expression levels of iNOS and eNOS in the ICA group were 1.8 and 2.7 times higher than those of the control respectively (Fig. 2C, 2E), and the protein expression levels of these two enzymes were also significantly increased compared to

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the non-stimulated control (Fig. 2D, 2F). However, these stimulatory effects of ICA on NOS activity, NO production and iNOS or eNOS expression nearly disappeared or completely blocked when L-NAME, a pharmacological inhibitor for NOS, was added together with ICA (Fig. 2). The addition of L-NAME also inhibited the increases in sGC expression level and cGMP content induced by icariin supplementation (data not shown).

ODQ inhibits the increased sGC and cGMP expression induced by ICA

Soluble guanylate cyclase (sGC) is a downstream enzyme targeted by NO. As shown in Fig. 3A, addition of ICA to the culture (which increased NO production as shown above) significantly increased the mRNA level of sGC. However, this effect was inhibited by the addition of ODQ (a specific inhibitor of sGC) in combination with ICA in the culture. Consistently, the sGC protein levels were increased significantly in ICA-treated cultures, which again were blocked with the presence of ODQ (Fig. 3B). In addition, since cGMP is downstream of sGC, we hypothesized that if the activity of sGC was blocked by ODQ, cGMP content should decrease as well. Confirming our hypothesis, the cGMP content in ICA group was found the highest among four groups ($P<0.01$ vs control group), and the effect of ICA in inducing cGMP was decreased significantly after the sGC in the upstream was inhibited by ODQ (Fig. 3C).

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PDE5 inhibits cGMP production and PKG expression induced by ICA

As shown in Fig. 4A, the cGMP production increased sharply in ICA group, and this induction effect of ICA on cGMP production was blocked by with the presence of cGMP inhibitor PDE5. To further verify the importance of the signal pathway in mediating ICA osteogenic activity, we examined whether the production of PKG (which is downstream of cGMP) could be down-regulated after cGMP is inhibited. Confirming our predictions, inhibition of cGMP production with PDE5 suppressed the expression of mRNA (Fig. 5B) and protein (Fig. 5C) of PKG (P<0.01 compared to ICA group).

Ly294002 inhibits p-AKT level, NOS activity, eNOS expression and NO production induced by ICA

To investigate whether PI3K/AKT pathway was associated with the osteogenic effect of ICA and NO production, we firstly examined the expression of phosphorylated AKT (p-AKT). As shown in Fig. 5A, the p-AKT expression level was increased significantly in the ICA group. However, when LY294002, a specific inhibitor of PI3K [47], was added together with ICA, the expression of p-AKT was greatly reduced (P<0.01 vs ICA group). Meanwhile, the total NOS activity and NO

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production were also significantly decreased (P<0.05) (Fig. 5D, 5E). The iNOS protein level was decreased, but no significant difference was found (Fig. 5B). The eNOS protein level, however, was found very sensitive to the addition of LY294002, and decreased to an even lower level than the control (Fig. 5C). The sGC protein levels and cGMP contents were also examined after the addition of LY294002, with the tendencies of their changes being similar with that of eNOS (data not shown). These results indicated the following signaling pathway in mediating the osteogenic effect of ICA: ICA activates the phosphorylation of AKT, and p-AKT promotes the expression and activity of NOS and thus increases the NO production. In this step, the induction of eNOS expression was more notable than that of iNOS, suggesting that eNOS is the main target of p-AKT in ICA-induced osteogenic activity.

All inhibitors involved in PI3K-AKT-NOS-NO-cGMP-PKG pathway inhibits osteogenic differentiation of rBMSCs induced by ICA

To investigate the role of the PI3K-AKT-NOS-NO-sGC-cGMP-PKG pathway identified above in mediating icariin-induced osteogenesis, treatment effects in rBMSCs of icariin in the presence, in a stepwise manner, of various key inhibitors of the pathway were analyzed on various osteogenesis markers. The intracellular alkaline phosphatase (ALP) activity was measured after 9 day of the osteogenic induction culture. As expected, ALP activity of the ICA-treated group was

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significantly higher than the control group (P<0.01) (Fig. 6A). When 100 μM L-NAME (a NOS inhibitor), 20 μM ODQ (a sGC inhibitor), 0.02 Unit/mL PDE5 (a cGMP inhibitor), or 50μM LY294002 (a specific inhibitor of PI3K) was supplemented, the PI3K-AKT-NOS-NO-sGC-cGMP-PKG signal pathway was blocked and the stimulatory effect of ICA on ALP activity was abolished. While four inhibitors blocked ALP induction caused by ICA supplementation, treatment alone with any of the four inhibitors induced no significant differences in ALP activity compared with control group.

The osteocalcin secretion was measured at the same time point with ALP assay (9 days after osteogenic induction) (Fig. 6B). The osteocalcin secretion of ICA-supplemented group was significantly higher than that of the control (P<0.01). The increases in osteocalcin secretion were abolished significantly after any of the four inhibitors of PI3K-AKT-NOS-NO-sGC-cGMP-PKG pathway was added (P<0.01 compared to ICA group).

The calcium deposition was measured after 12 day of the osteogenic induction culture and again the ICA-treated group yielded the highest calcium deposition among all the groups (Fig. 6C) (P<0.01 compared to the control group), and this induction effect was significantly blocked by any of the four inhibitors tested for NO-cGMP-PKG signaling pathway (P<0.01 compared to ICA group).

The calcified nodules were analyzed by staining of Alizarin Red-S after 12 days of the osteogenic culture, and the results showed a similar tendency with calcium

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deposition data (Fig. 7). Among all the groups, the ICA-treated group had the highest number and largest area of mineralized nodules, confirming the stimulatory effect of ICA in the osteogenic differentiation of rBMSCs (Fig. 7A) (P<0.01 compared with the control group). However, after any of the four inhibitors of PI3K-AKT-NOS-NO-sGC-cGMP-PKG signaling pathway was added, the induction effect of ICA in the staining area (Fig. 7B) and intensity (integrated optical density or IOD) (Fig. 7C) of calcified nodules was blocked significantly (P<0.01 compared to the ICA group).

Ability of the physiologically achievable concentration of icariin in activating this signal pathway

The above signal pathway studies have been carried out with icariin at 10^{-5} M, a concentration found by us to induce the strongest ALP activity in rBMSCs in vitro. To investigate whether the in vivo physiologically achievable concentration of icariin (10^{-6} M) is effective in activating this signal pathway, we have also supplemented icariin at 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M, respectively, and have examined the treatment effects on total NOS activity and the expression levels of phosphorylated AKT (p-AKT), two important markers of the signaling pathway activation. As shown in Fig. 8, the NOS activity (Fig. 8A) and p-AKT expression levels (Fig. 8B) were both increased by icariin in a dose-dependent manner, and 10^{-6} M icariin induced

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significantly higher NOS activity ($P<0.01$) and a p-AKT expression level ($P<0.01$) compared to the non-stimulated control group.

Discussion

Previously, despite unclear action mechanism, we and others have demonstrated that icariin (ICA), extracted from Epimedium, is a potent stimulator of osteogenic differentiation in vitro and it can prevent bone loss in rat osteoporosis models [14,15,17,43]. As a result, it is a promising candidate as a natural, alternative way of preventing bone loss and treating osteoporosis. In addition, it would be an attractive candidate due to its low toxicity/side effect profiles in comparison to the potential and in some cases serious adverse effects of existing drugs for treating osteoporosis [20]. Using tissue culture models and osteogenic markers (levels of alkaline phosphatase activity, osteocalcin synthesis and calcium deposition), our previous studies have confirmed the stimulating effect of ICA on osteogenic differentiation in rat bone marrow stromal cells (rBMSCs) and rat calvarial osteoblasts (ROB) [48,49]. Here, we also showed that icariin can dose-dependently promote osteogenic differentiation of rBMSCs. Despite the above previous and current in vivo and in vitro studies demonstrating efficiency of ICA in promoting osteogenesis, its action mechanisms are still unclear. In the current study, we

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identified that icariin promotes osteogenic differentiation of rBMSCs via enhancing activation of the PI3K-AKT-eNOS-NO-sGC-cGMP-PKG signal pathway.

Recently, an accumulating body of evidence has emerged to suggest that NO plays an important role as a paracrine and autocrine mediator of bone cell activity in response to diverse stimulus [23,24]. NO has emerged as a potent regulator useful in alleviating estrogen deficiency bone loss [50-52]. Some reports suggested that long-term oral treatment with icariin-enriched fraction and/or pure icariin might improve the erectile function of castrated rats, and this effect of icariin was related to the NOS activity and NO content [26]. In addition, they also found icariin could improve erectile function in streptozotocin-induced diabetic rats by stimulating the NOS expression and increasing the contents of NO and cGMP in the penis of diabetic rats [27]. Another study found that icariin at 10^{-8} M increased the proliferation and matrix mineralization of primary osteoblasts and promoted NO synthesis, and that expression of BMP-2, SMAD4, Cbfα1/Runx2, and OPG genes was up-regulated and that of RANKL down-regulated, suggesting that the osteogenic effects of icariin may be through the induction of BMP-2 and NO pathways [21]. Icariin had also been shown to stimulate angiogenesis by activating the MEK/ERK- and PI3K/AKT/eNOS-dependent signal pathways and enhance NO production in human umbilical vein endothelial cells by androgen receptor-dependent activation of eNOS [28, 53]. Although the relationship between icariin and NO has been proved, the factors in the upstream and downstream of NO during the process of osteogenesis

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have not been clarified in these studies. The present study represents the first report demonstrating that icariin stimulates the osteogenic differentiation of bone marrow stromal cells via activating the PI3K-AKT-eNOS-NO-sGC-cGMP-PKG signal pathway.

In our experiments, total activity or expression levels of NOS, iNOS, eNOS and NO were found to be increased during the osteogenesis process of rBMSCs induced by ICA, indicating that ICA enhanced the activity of total NOS, thereby increasing the content of NO. Confirming the role of this pathway in mediating ICA's osteogenesis effect, L-NAME, the inhibitor of iNOS and eNOS, significantly decreased total NOS activity, NO production and ICA's osteogenic outcomes. These data confirmed the important role of ICA in NO signal pathways. Further analyses showed that ICA significantly promotes the expression of sGC and cGMP during osteogenesis, while the ICA-induced osteogenesis markers was substantially blocked after ODQ being added into the cultures of rBMSCs, suggesting sGC plays a role in signaling pathway. In the analysis of the NOS-NO-sGC-cGMP-PKG pathway, our results revealed that ICA-induced osteogenesis involves cGMP and PKG. cGMP is the second messenger which can be broken down by phosphodiesterase-5 (PDE5) specifically. While ICA increased the contents of cGMP and PKG in rBMSCs, the induction of both of these markers was blocked after the addition of PDE5. Again, these findings suggest that the NOS-NO-sGC-cGMP-PKG pathway mediates positive effect of ICA in osteogenic differentiation of rBMSCs.

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To identify whether PI3K/AKT is upstream of NOS, we examined the p-AKT expression, NOS activity, NO production, iNOS and eNOS expression in the presence of ICA and LY294002, a specific inhibitor of PI3K. The results showed that ICA activates the phosphorylation of AKT, and when p-AKT expression was blocked by LY294002, the total NOS activity, NO production and eNOS were also reduced notably. Taken together, our studies provide strong evidence that the PI3K-AKT-eNOS-NO-sGC-cGMP-PKG pathway is essential in mediating the osteogenic effect of ICA. Furthermore, while our data have shown that the 10^{-5} M icariin concentration used was able to activate the above signaling pathway to the highest degree, our dosage response data also showed that icariin at 10^{-6} M (the physiologically achievable concentration of icariin in vivo 10^{-6} M [59]), is also effective in promoting osteogenic differentiation of rBMSCs in vitro through activating the this same pathway.

In this experiment, iNOS expression was increased significantly by ICA, indicating that iNOS contributes a part of the NO production. However, the combination addition of LY294002 and ICA only significantly inhibited the expression of eNOS, but not the expression of iNOS, demonstrating the possible existence of another pathway to modulate the expression and activity of iNOS. It is known that both eNOS and iNOS are important regulators of bone remodeling. While eNOS is constitutively expressed, producing small quantities of NO, and mediates bone formation, iNOS is expressed in response to different stimuli associated with

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inflammatory responses, producing NO at a high concentration, and usually induces bone resorption [54]. Since LY294002 completely abolished the effects of ICA on total NOS activity and NO production, its significant effect in abolishing the ICA activity on expression of eNOS but not iNOS in this experiment suggests that iNOS may have a lesser contribution to the total NOS activity and NO production during icariin-stimulated osteogenic differentiation.

Further studies are still required to investigate how icariin activates the phosphorylation of AKT, or which signal molecules lies in the upstream of PI3K-AKT-eNOS-NO-sGC-cGMP-PKG pathway. A possible pathway is that icariin binds with estrogen receptor (ER) as a phytoestrogen, and the activated ER further activates the phosphorylation of AKT. However, in our previous studies, we had obtained some evidence suggesting that icariin exerts osteogenic activity through a non-classical estrogenic pathway. We compared icariin with genistein, an isoflavone with the highest affinity to ERs among the isoflavones contained in soybean [55, 56], and found that icariin is more potent than genistein in promoting osteoblast differentiation and mineralization in vitro [43]. The mechanism of higher osteogenic activity of icariin was found to be associated with the prenyl group on C-8 of icariin [57]. Similarly, the comparison between 8-prenylnaringenin and naringenin further demonstrated that 8-prenyl-flavonoids represent a class of natural flavonoids with higher bone-protective properties [42，58]. Further studies are required to investigate whether the 8-prenyl group of icariin is involved in the activation of AKT

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phosphorylation and PI3K-AKT-NOS-NO-sGC-cGMP-PKG pathway.

In summary, the present study proved our hypothesis that local NO levels mediate the beneficial effects of icariin, with PI3K/AKT and NO-sGC-cGMP-PKG pathways lying in the upstream and downstream of NO pathway respectively, and that p-AKT mainly activates eNOS but not iNOS. These findings indicate that icariin stimulates the osteogenic differentiation of rBMSCs partly through activating the PI3K-AKT-eNOS-NO-sGC-cGMP-PKG signal pathway. However, further studies are needed to investigate the mechanism how icariin activates PI3K and to identify the target/receptor molecule of icariin on the cell surface.

Acknowledgements

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Figure legends:

Table 1. The primer sequences for Real Time RT- PCR

Figure 1. Phenotypic and purity characterization of rat bone marrow stromal cells (rBMSCs) (A), and the dose-dependent manner of icariin in stimulating osteogenic differentiation of rat bone marrow stromal cells (B). In phenotypic and purity analysis, the cells were incubated with fluochrome-labeled primary antibodies against CD44, CD45, CD90 and corresponding isotype control, and the stained cells were immediately detected by flow cytometric analysis (A). When the dosage effect of icariin was assayed, the cells grown to 80% confluence were cultured in osteogenic medium for 9 days with various concentrations of icariin, with ALP activity being measured (B). $^{*}P < 0.05$, $^{**}P < 0.01$ vs control.

Figure 2. Effects of ICA and L-NAME on total NOS activity, expression of iNOS and eNOS, and NO production after different time of osteogenic induction culture in rat bone marrow stromal cells (rBMSCs). The intracellular total NOS activity (A), NO contents in culture medium (B), mRNA and protein expression of iNOS (C, D) and eNOS (E, F) were examined after the cells were treated for 12 h (A, B, C, E) or 24h (D. F) with 10^{-8} M ICA, 10^{-4} mol/L L-NAME or combination of them. The relative

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optical density of western blot bands was measured by Image-Pro Plus6.0 (D, F). $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs* ICA.

Figure 3. Inhibition of the NO-sGC-cGMP-PKG pathway with ODQ blocking ICA-induced sGC expression and cGMP production during osteogenic induction culture of rBMSCs. A: mRNA expression of sGC of cells treated for 12 h in the presence of 10^{-5} mol/L ICA and/or ODQ. B: sGC Western blotting analysis with cells cultured for 24 h in the presence of ICA and/or ODQ. C: The cGMP content after the signal pathway was inhibited with ODQ at 12 h. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs* ICA.

Figure 4. PKG expression and cGMP contents in rBMSCs at 12 h after osteogenic culture in the presence of absence of 10^{-5} mol/L ICA and cGMP inhibitor PDE5 at 0.02 Unit/mL. A: cGMP concentration as examined by cGMP Enzyme Immunoassay Kit. B: The relative mRNA level of PKG as measured by Real-Time RT-PCR. C: The relative content of PKG as determined by Western blotting. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs* ICA.

Figure 5. Effects of ICA and LY294002 (a specific inhibitor of PI3K) on p-AKT, iNOS and eNOS expression, total NOS activity and NO production. The expression of p-AKT (A), iNOS (B), eNOS (C) was examined by Western blotting after rBMSCs

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were treated with 10^{-5} mol/L ICA, 50μM LY294002 or their combination. The relative optical density of Western blot bands was measured by Image-Pro Plus6.0 (A, B, C). The total NOS activity (D) and NO contents (E) were measured after 12 h of above treatments. $^{*}P < 0.05$，$^{**}P < 0.01$ vs control; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs* ICA.

Figure 6. ALP activity, osteocalcin secretion, and calcium deposition of osteogenic induction culture of rBMSCs treated with 10^{-8}M ICA, 100 μM L-NAME (a NOS inhibitor), 20 μM ODQ (a sGC inhibitor), 0.02 Unit/mL PDE5 (a cGMP inhibitor), and 50μM LY294002 (a specific inhibitor of PI3K). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs* ICA.

Figure 7. Effects of ICA and L-NAME (a NOS inhibitor), ODQ (a soluble guanylate cyclase inhibitor), PDE5 (a cGMPinhibitor) and LY294002 (a specific inhibitor of PI3K) on calcified nodules of rBMSCs on 12th day of the osteogenic induction. A: Representative images of mineralized bone nodules formed. B: Comparison on the area of calcified nodules among different groups. C: The intensity optical density (IOD) comparison among the different groups. $^{**}P < 0.01$ between two groups.

Figure 8. The dosage effects of ICA on total NOS activity and expression level of p-AKT. ICA was supplemented at 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M, respectively, and the intracellular total NOS activity was measured after 12 h (A). The p-AKT expression

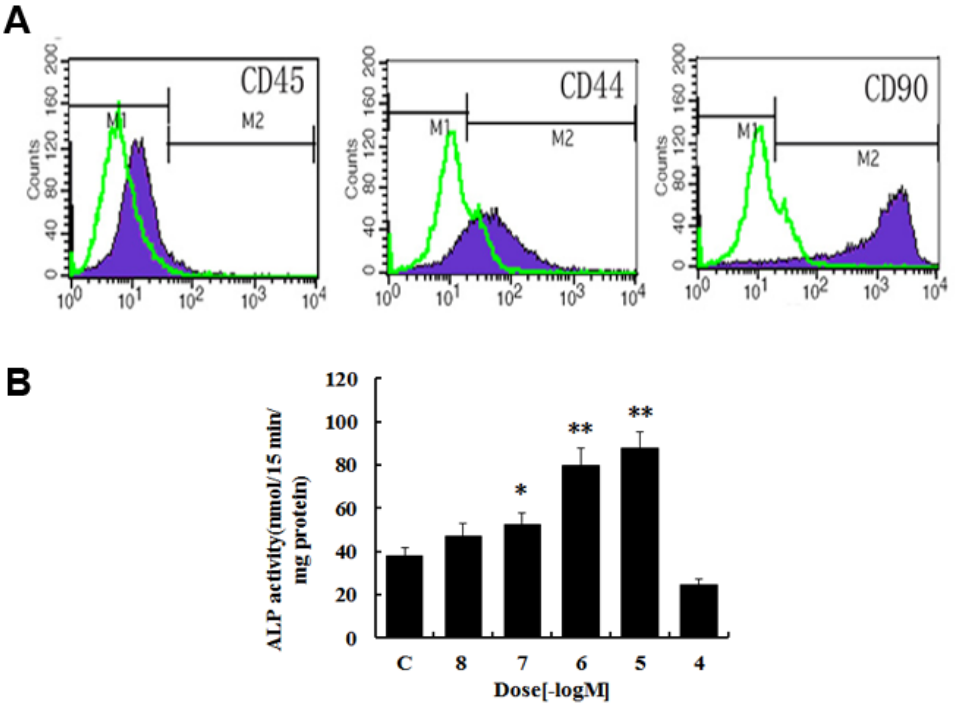
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was examined by Western blotting after 24 h of above treatments. The relative optical density of Western blot bands was measured by Image-Pro Plus6.0 (B). $^{*}P < 0.05$, $^{**}P<0.01$ vs control.

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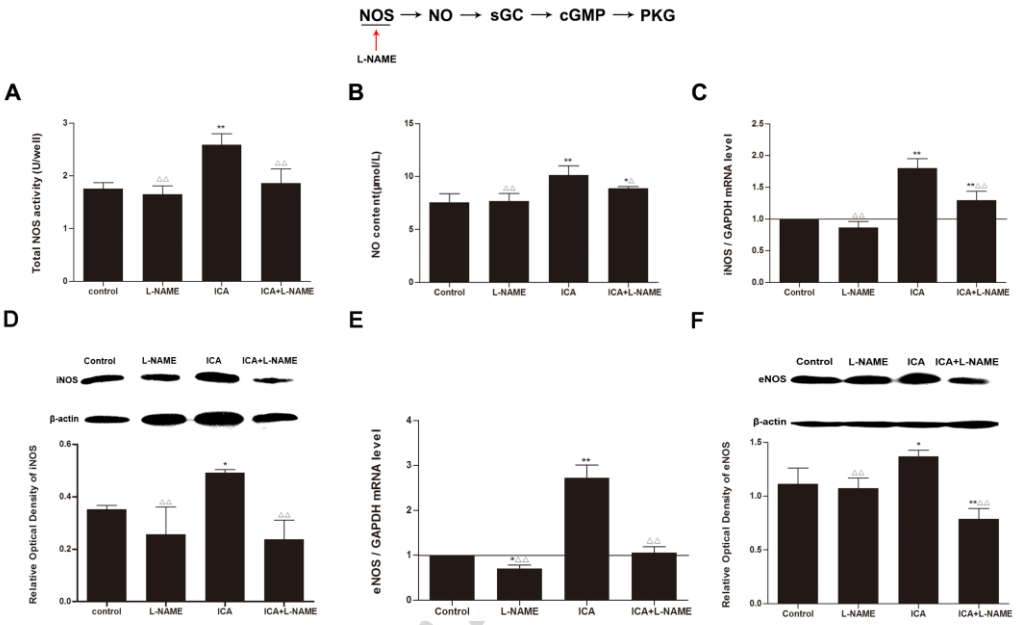
Figure 1



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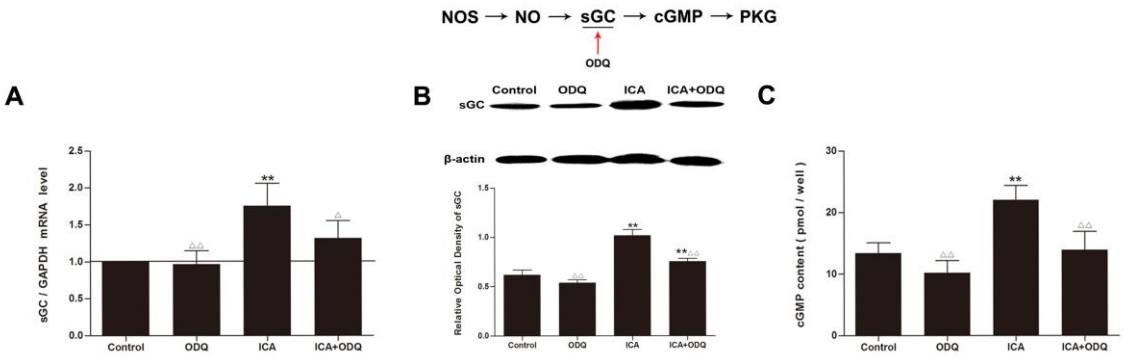
Figure 2



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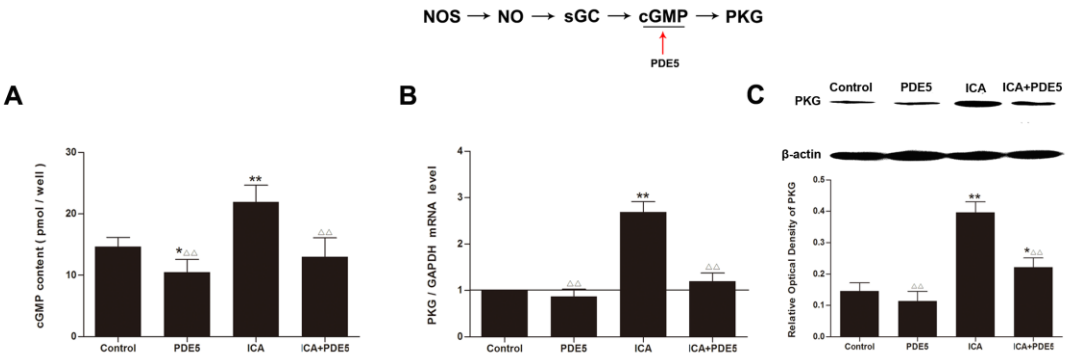
Figure 3



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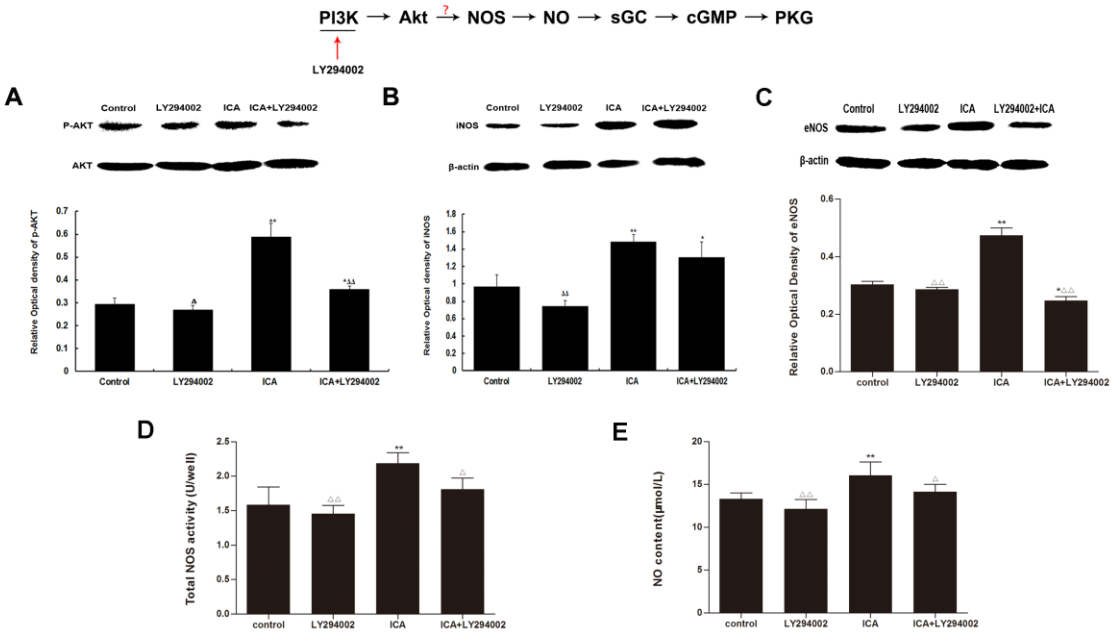
Figure 4



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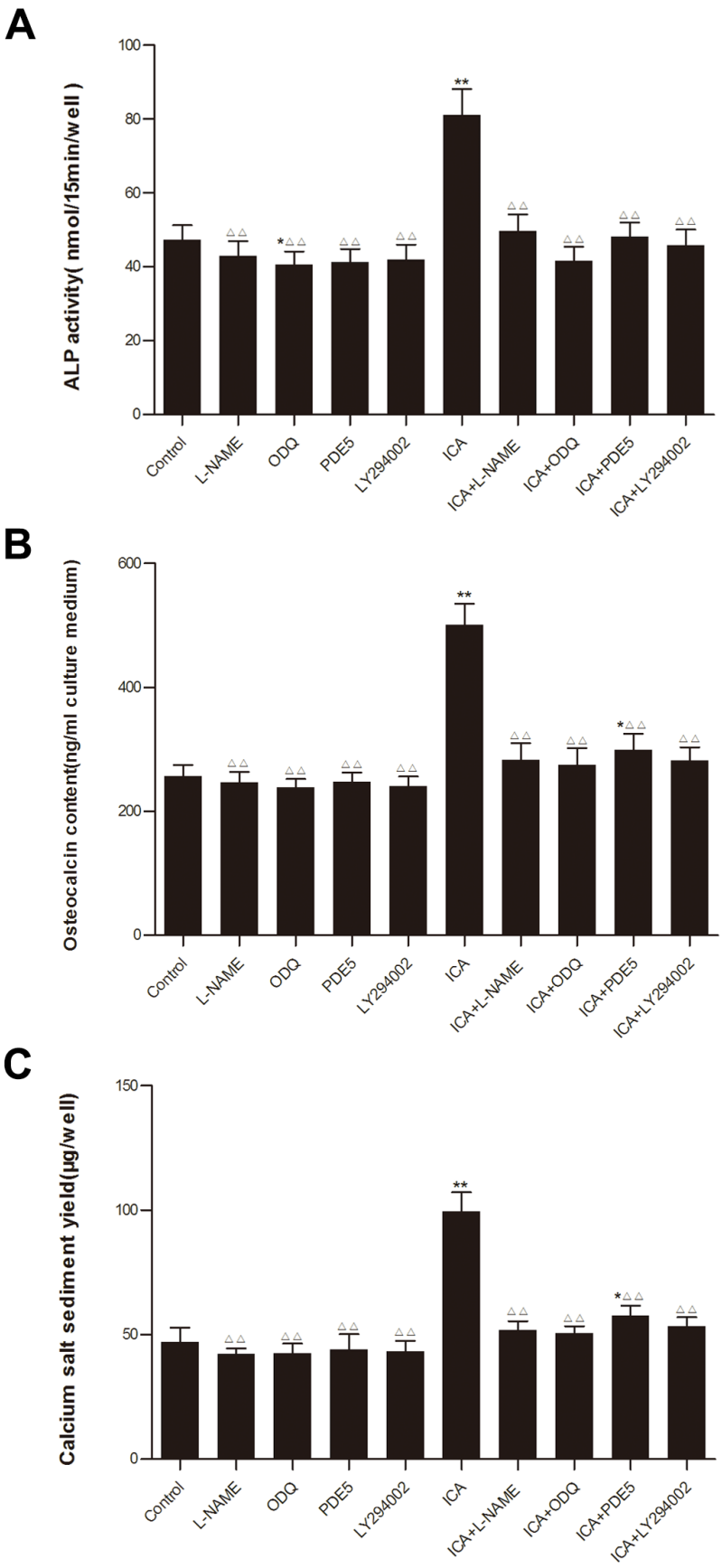
Figure 5



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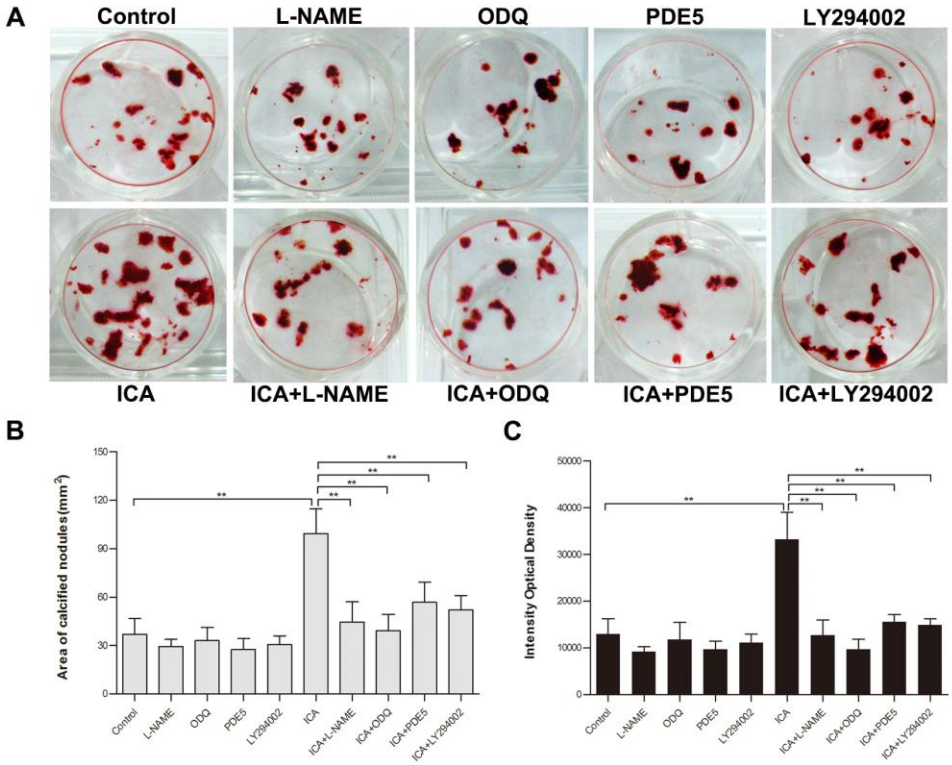
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Figure 6



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Figure 7



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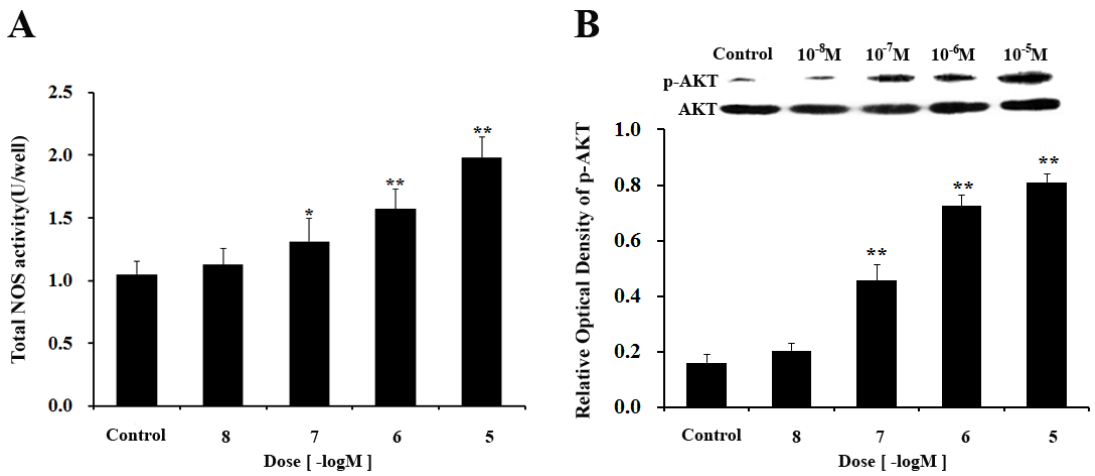


Figure 8

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Table 1. The primer sequences of Real Time RT- PCR

Gene	GeneBank No.	Primer sequence		Product length/bp
iNOS	NM_012611.3	Forward	5'- CTCACTGTGGCTGTGGTCACCTA -3'	101
		Reverse	5'- GGGTCTTCGGGCTTCAGGTTA -3'	
eNOS	NM_021838.2	Forward	5′-GCATACCCGCACTTCTGT-3′	108
		Reverse	5′-GCCTTGGCATCTTCTCCC-3′	
sGC	NM_017090.2	Forward	5′- CGCTTTGACCAACAGTGTGGA -3′	127
		Reverse	5′- GGGCCATCAGTGCTATCTGGA -3′	
PKG	NM_001105731.2	Forward	5′- AGGAGCACATCCGCTCAGAGA -3′	122
		Reverse	5′- ACCTAGGCAAGCTTCCATCAACA -3′	
GAPDH	NM_017008.3	Forward	5′- TATCGGACGCCTGGTTAC -3′	140
		Reverse	5′- CTGTGCCGTTGAACTTGC -3′	

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Highlights

- Icariin is a prenylated flavonoid with strong osteogenic activity and has potential application in preventing bone loss.
- We hypothesized that icariin osteogenic action is related to activation of NO and PI3K/AKT pathways.
- Icariin potentiated osteogenic differentiation of rat bone marrow stromal cells through enhancing alkaline phosphatase activity, osteocalcin expression, and mineralization.
- The osteogenic effect of icariin involves activation of the PI3K-AKT-eNOS-NO-cGMP-PKG signal pathway.

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