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Original Article

Effects of icariin on osteogenic differentiation of bone marrow stromal cells in beagle canine

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Abstract: Icariin (ICA), as the main active component in perennial herb Epimedium, displays a wide range of pharmacological and biological effects, including enhanced sexual function, immune regulation, anti-inflammatory, and anti-osteoporosis. In this study, the effect of ICA on osteogenic differentiation of bone marrow derived stromal cells (BMSCs) from a Beagle dog was assessed. BMSCs were Isolated from the canine bone marrow, cultured and identified by applying morphology and multi-lineage differentiation assays. Cell counting kit-8 (CCK-8) assay showed that ICA significantly promoted BMSC proliferation in a dose-dependent manner. Alkaline phosphatase (ALP) activity assay showed that ICA can increase ALP activity in a dose-dependent manner. Furthermore, RT-PCR and Western-blot indicated that ICA upregulated osteogenic-related genes and proteins. Alizarin red staining demonstrated the induced calcium deposition in BMSCs treated with 10^{-6} mol/L ICA for 21 days. Overall, these findings clearly indicate that ICA is able to promote the osteogenic differentiation of BMSCs in beagle dogs. The optimal ICA concentration of 10^{-6} mol/L may be appliable in bone tissue engineering.

Keywords: Bone marrow stromal cells (BMSCs), icariin, osteogenic differentiation, canine, bone tissue engineering

Introduction

Multiple pathological processes can cause different kinds of bone defects, including trauma, inflammation and bone tumors. Slight bone defects can self-resolve, but large volume bone defects remain a huge challenge in the field of Orthopedics and oral implantology. Autogenous bone graft, allogenic bone transplantation and artificial bone filling are the traditional repair methods for large volume bone defects [1, 2]. Thanks to its conductivity and inductive activity, autogenous bone graft is considered as the gold standard of bone defect repair. However, this technique has complications such as secondary traumatization of the donor site and infection [3]. Allogenic bone transplantation has its own limitations, including limited resources and immunological rejection [4], and artificial bones lack inductive activity. It is expected that bone tissue engineering may solve these clinical problems [5, 6]. Bone tissue engineering is an interdisciplinary science, which underlies biology and engineering, developing viable substitutes for bones. It can be used to repair, maintain and improve the function of bones. Bone tissue engineering materials comprise osteogenesis seed cells, bone induced growth factors and support materials with bone conduction activity.

Bone marrow mesenchymal stem cells (BMSCs) are easily obtained, with multi-lineage differentiation potential; they can differentiate into chondrocytes, osteocytes, fat cells and endothelial cells [7]. Therefore, they are considered as ideal seed cells for bone tissue engineering to repair bone defects [8]. For bone regeneration or bone repair, mesenchymal stem cells derived from the bone marrow give rise to osteogenesis progenitor cells, which in turn generate osteoblasts that undergo differentiation before the final formation and mineralization of bone matrix [9].

Growth factors are often used in the field of bone regeneration, to improve and accelerate the bone healing process. BMPs count among

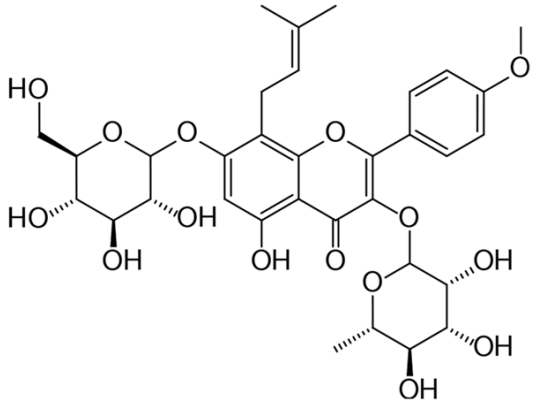

Figure 1. Chemical structure of icariin. It is a prenyl flavonoid glycoside with a glucosyl group on C-3; a rhamnosyl group on C-7; a methoxyl group on C-4; and a prenyl group on C-8 position.

the most important growth factors. In animal models and clinical trials, both BMP-2 and BMP-7 can significantly promote bone formation [10-12]. However, the clinically used effective dose of BMP is too high for repair [13, 14]; in addition, materials containing BMP tend to have a certain rate of failure, thus drawing attention to their cost and safety [15, 16]. High costs and rapid degradation are the main drawback of BMPs, limiting their clinical application [17]. Therefore, developing new alternative agents with higher efficacy and lower cost is urgently required in bone regenerative medicine.

Epimedium is one of the most widely used Chinese herbal medicines. Its main active ingredient, icariin, has extensive pharmacological effects including sexual function enhancement and osteogenesis induction, as well as anti-cancer, anti-oxidation, anti-depressant and estrogen-like activities [18, 19]. In the field of bone regeneration, Icariin has been shown to improve osteogenesis *in vitro* by regulating endogenous BMP-2 and inducing nitric oxide (NO) synthase [20-22]. In the other hand, Epimedium can inhibit MAPK signaling pathways to decrease the amounts of osteoclast cells [23] and enhance the ratio of OPG to RANKL [24]. Compared with BMPs, Epimedium glycoside is of low cost with fewer side effects [25]. Because of its rich source, low price and good bone regeneration, Epimedium glycoside has great clinical application prospect.

The function of icariin in BMSCs of Beagle dogs remains unclear yet. In this study, we firstly isolated and purified primary BMSCs from Beagle dog's ilium by morphology and multi-lineage differentiation; in addition, the optimal concentration of icariin was determined by detecting its effect on the proliferation and differentiation of BMSCs. Then, we evaluated the effects of Icariin on osteogenesis related genes and proteins. Finally, BMSCs were assessed for calcium nodules by alizarin red staining, after 21 days of culture in appropriate icariin concentrations. Our findings indicated that icariin can significantly promote the osteogenesis differentiation of BMSCs, providing a theoretical basis for further study of icariin in promoting bone defect around the dental implant.

Materials and methods

Major reagent and equipment

Icariin (C33H40O15, Mw: 676.67, purity >98%) was purchased from Tauto Biotech (Shanghai, China) (**Figure 1**). Low-glucose DMEM medium was obtained from Hyclone (US).Fetal bovine serum (FBS), Trypsin-EDTA digestion buffer and penicillin-streptomycin were purchased from Gibco (US). DMSO and PBS were purchased from Sigma (US). Osteogenic differentiation medium and adipogenic differentiation medium were purchased from Cyagen (China). Cell Counting Kit-8 for cell proliferation was purchased from DOJINDO (Japan). Alkaline phosphatase activity assay kit was purchased from Jiancheng (China). RNA extraction kit and reverse transcription kit were purchased from TakaRa (Japan). Primers were synthesized by Sangon (China). Western blotting reagent was purchased from Beyotime (China). Rabbit anti-dog Runx-2 antibody, rabbit anti-dog OCN monoclonal antibody were obtained from Abcam (US). Fluorescent quantitative PCR cycler (7900 Fast) was produced by ABI (US). Automatic microplate reader and Western-blot instruments were produced by Bio-Rad (US).

Isolation and culture of BMSCs

A male Beagle aged 1.5 years (20 kg) was obtained from the animal shelter of the Ninth People's Hospital, Shanghai Jiaotong University Medical College. The Institutional Animal Care and Use Committee of the Ninth People's Hospital, Shanghai Jiaotong Jiaotong University, approved the protocol used in animal experiments. About 6 ml of bone marrow of the dog

Table 1. Primer sequences of genes for real-time PCR

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
β-actin	GCAAGGACCTCTATGCCAACA	GAAGCATTTGCGGTGGACG
Osterix	CTGCGGGACTCAACGACACT	AGGAGGGTACTCATTGGCATAGC
bFGF	AAGAGCGATCCCCACGTCAA	TTCGTTTCAGTGCCACATACCA
Runx-2	TCCAGACCAGCAGCACTCCATA	TTCCATCAGCGTCAACACCATC
OCN	TCACAGACCCAGACAGAACCG	AGCCCAGAGTCCAGGTAGCG

was extracted from the iliac bone, and cultured in 5% CO_2 incubator at 37°C. Half medium was changed after 2 days. Suspension cells were removed and the adherent ones collected; at 70%~80% confluency, 0.25% trypsin-EDTA digestion was carried out for 2 min. The cells of the P4 generation were used in this study.

Multi-lineage differentiation of BMSCs in vitro

To detect the multi-lineage differentiation potential of BMSCs, they were cultured in osteogenic inducing medium and adipogenic inducing medium. The osteogenic inducing medium was changed once every 3 days; 3 days after induction with A solution in the adipogenic induction group, B solution was added for 1 day. After repeating this cycle 5 times, the culture medium was removed, and cells were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, and stained with alizarin red and oil red stain. The color was observed and analyzed by optical microscopy.

Cell counting kit-8 (CCK-8) assay for cell proliferation

To assess the effect of icariin on beagle BMSC proliferation, changes in the number of living cells were quantified by the CCK-8 assay. P4 generation BMSCs were seeded into 96 well plates at 3000 cells/well; after 24 h of incubation, adherent BMSCs were treated with complete medium containing 0 (control group), 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} mol/L ICA, in 6 replicates. After 1, 3, 5, and 7 d, the culture medium was removed, followed by addition of 100 μl complete culture medium containing 10 μl CCK-8 to each well. After further incubation in presence of 5% CO_2, at 37°C for 1.5 h, optical density (OD) was measured at 450 nm.

Alkaline phosphatase (ALP) activity assay

BMSCs were seeded into 6 well plates at a density of 2×10^5 cells/well; after culture for about 24 h, complete culture medium containing 0 (control group), 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} mol/l ICA, respectively, was added to each well. OD values at 520 nm were measured at 1, 3, 5, and 7 d, as directed by the guidelines of the alkaline phosphatase kit; meanwhile, total protein in various samples was determined using the BCA method. ALP activity was determined as absorbance at 520 nm per milligram of total cellular protein.

RNA extraction and real-time quantitative RT-PCR

To detect the effect of Icariin on osteogenic related genes in BMSCs, the cells were treated with 10^{-6} mol/l ICA. Total RNA was extracted by TRIzol Reagent at 3, 5, and 7, respectively, and by spectrophotometry; the ratio of absorbance at 260 to 280 nm (A260/A280) was used to assess RNA purity. Reverse transcription was completed with 1 μg of total RNA in a final volume of 20 μl. The gene expression levels of osterix, bFGF, Runx-2, and OCN were determined by real-time quantitative PCR with 1 μl of cDNA in a 10 μL reaction volume. Data were normalized to β-actin. Standard curves were plotted for semi-quantitative analysis by the 2-ΔCt method. All experiments were performed in triplicate, with the primers listed in **Table 1**.

Cellular protein isolation and western blot

The protein expression levels of osteogenic factors were analyzed in ICA-treated BMSCs at 4, 6, and 8 d; osteocalcin (OCN) and runt-related transcription factor 2 (Runx-2) were assessed. Total protein was collected from cultured cells at 4, 6, and 8. Briefly, after cell lysis, protein concentrations were measured. Equal amounts of total protein were separated in duplicate by 10% SDS-PAGE and transferred onto 0.45 mm polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in Tween Tris-buffered saline (TBST) at room temperature on a shaker for 1 h, the membranes were incubated with anti-OCN and anti-Runx-2 primary antibodies overnight at 4°C. The membranes were then washed three times with TBST and incu-

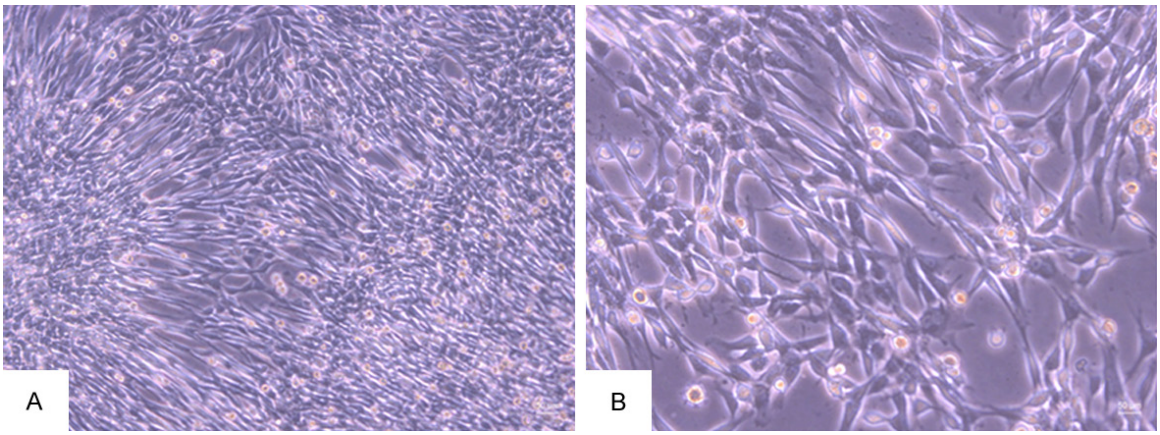

Figure 2. Morphology of BMSCs. (A) Passage 0 (P0) cells were cultured for one week, after which they were trypsinized and passaged as P1 cells. (B) At passage 1, the cells were adherent and showed the typical spindle-like, long and elongated fibroblastic shape. Magnification ×100 (A), ×200 (B).

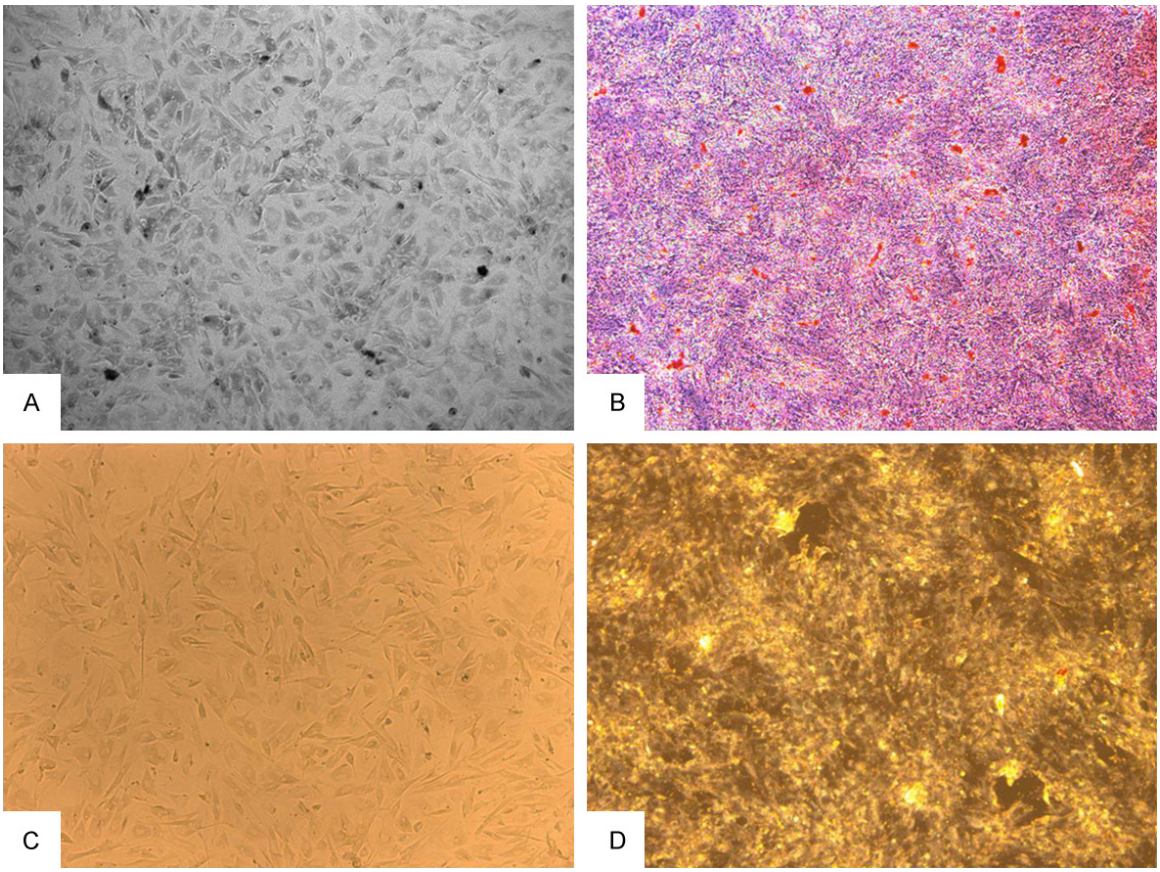

Figure 3. Light micrographs depicting the multi-lineage differentiation potential of BMSCs. (A, B) Mineralization in the osteogenic differentiation of BMSCs. Calcium nodules formed with BMSCs cultured in the osteogenic medium (B) compared with complete medium (A), as indicated by Alizarin red staining. Magnification ×100. (C, D) Adipogenic differentiation of BMSCs. Lipid droplets formed with BMSCs cultured in the adipogenic medium (D) compared with complete medium (C), as shown by Oil red O staining. Magnification ×100.

bated for 2 h with Horseradish peroxidase-labeled secondary antibodies at room temperature. Relative protein levels were obtained based on β-actin amounts.

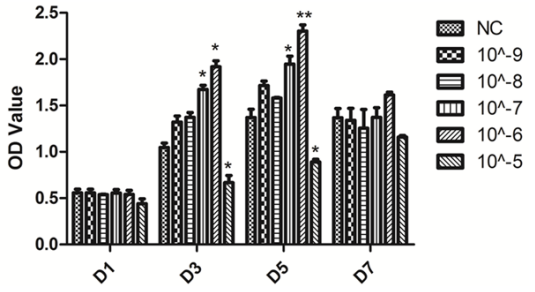

Figure 4. Effect of icariin on BMSC proliferation measured by Cell Counting Kit-8. The cells were incubated with icariin (10^{-9} M to 10^{-5} M) for 1, 3, 5 and 7 days, respectively. Complete medium served as control. Data are mean ± SD. *P<0.05 and **P<0.01 indicate statistically significant differences between the indicated group and the control group (blank) at the same time point.

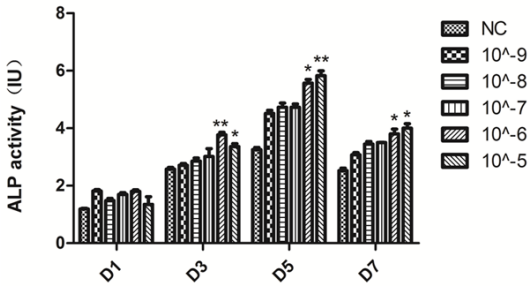

Figure 5. Icariin induces alkaline phosphatase (ALP) activity during the osteogenic differentiation of BMSCs. BMSCs treated with Icariin (10^{-9} M to 10^{-5} M) for 1, 3, 5 and 7 days, respectively, were lysed for ALP activity assessment.

ALP and ARS staining

According to CCK-8 and ALP results, 10^{-6} mol/l ICA was selected for BMSC treatment. P4 generation of BMSCs were plated on 3.5 mm culture dishes at a density of 3×10^5/wells and cultured in L-DMEM at 37°C in a humidified incubator containing 5% CO_2. Next, the cells were submitted to alkaline phosphatase (ALP)- and Alizarin Red-S (ARS) staining at treatment days 7 and 21, respectively, with 10^{-6} mol/l ICA. To prepare cells for ALP *staining*, they were washed three times with PBS and fixed in 4% paraformaldehyde for 30 min. The cells were then stained with ALP *staining solution* for 30 min at room temperature. For ARS assessment, after washing and fixation, the cells were stained with ARS for 3-5 min at room temperature.

Statistical analysis

All experiments were repeated at least three times, and similar results were obtained, Data are expressed as mean ± standard derivation, SPSS 20 statistical software was used to process the results, Unless stated otherwise, statistical significance was determined using Student's t-test and statistical significance was achieved when the *p* value is <0.05.

Results

Morphology and multi-lineage differentiation of BMSCs

After three days culture of whole bone marrow, the morphology of cells adherent to the plate is not easily observable by inverted microscopy, because of floating cells such as red blood cells. With gradual clarification of the culture medium, adherent polygonal, spindle shaped, clumped cells became visible (**Figure 2A**, **2B**). In the osteogenic induction medium, BMSCs began to fuse and aggregate. After 21 days, compared with the negative control group, more mineralized nodules were observed in the osteogenic differentiation group, and red nodules of different sizes and shapes were observed after alizarin red staining (**Figure 3A**, **3B**). Compared with the negative control group, formation of lipid droplets was observed after BMSC culture in the adipogenic differentiation medium (**Figure 3C**, **3D**).

ICA promotes the cell proliferation of BMSCs

Cell proliferation was detected by CCK-8. As shown in **Figure 4**, in addition to the 1×10^{-5} mol/l ICA group, the OD values of the remaining treatment groups, including the 1×10^{-7} and 1×10^{-6} mol/l groups, were higher than control values. At 3 and 5 d, OD values showed significant differences compared with the blank control group ($P<0.05$). OD in the 1×10^{-5} mol/l group was always lower than that control values, indicating a significant inhibition of cell proliferation (P<0.05).

ICA promotes the osteogenic differentiation of BMSCs

Alkaline phosphatase is an early marker of osteoblast differentiation. As shown in **Figure 5**, at 3, 5, and 7 d, the 1×10^{-6} and 1×10^{-5} mol/l in ICA groups showed increased ALP activity in BMSCs (P<0.05) compared with the NC group,

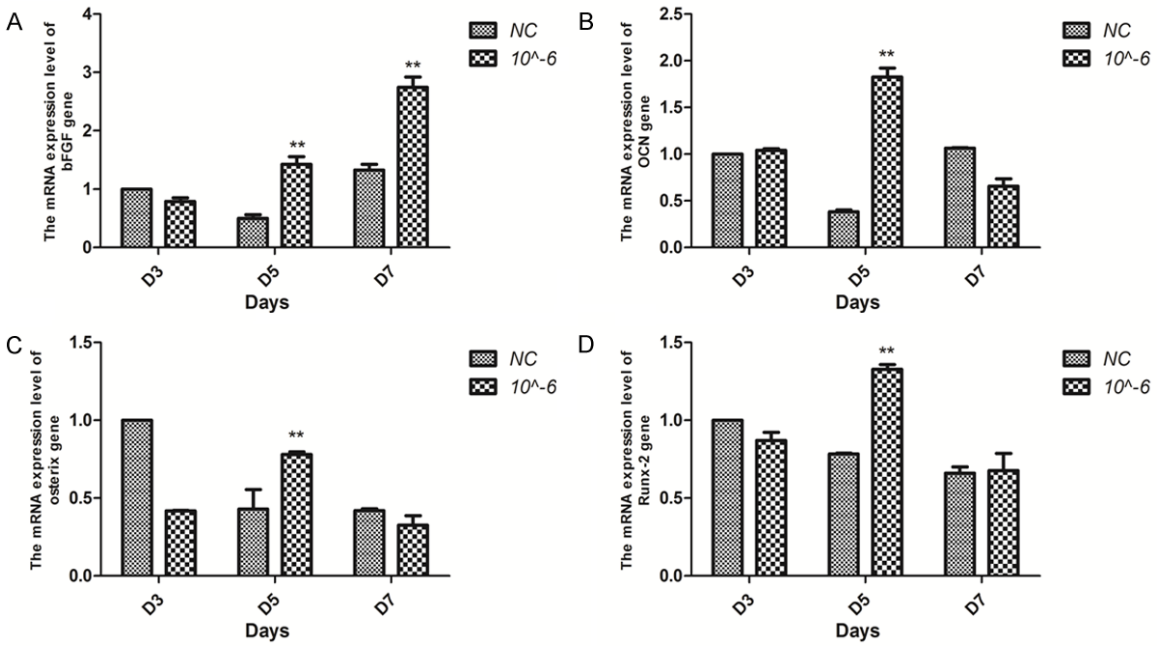

Figure 6. Changes of mRNA expression levels of osteogenesis-related genes at 3, 5, and 7 days, respectively, after treatment with 10^{-6} M ICA, as examined by real-time PCR. A. Basic fibroblast growth factors (bFGF); B. Osteocalcin (OCN); C. Osterix; D. Runx-2. All experiments were carried out in triplicates, and data are mean ± SD. *P<0.05 and **P<0.01 indicate statistically significant differences between the experimental (treated with 10^{-6} M icariin) and control group (no icariin) groups at the same time point.

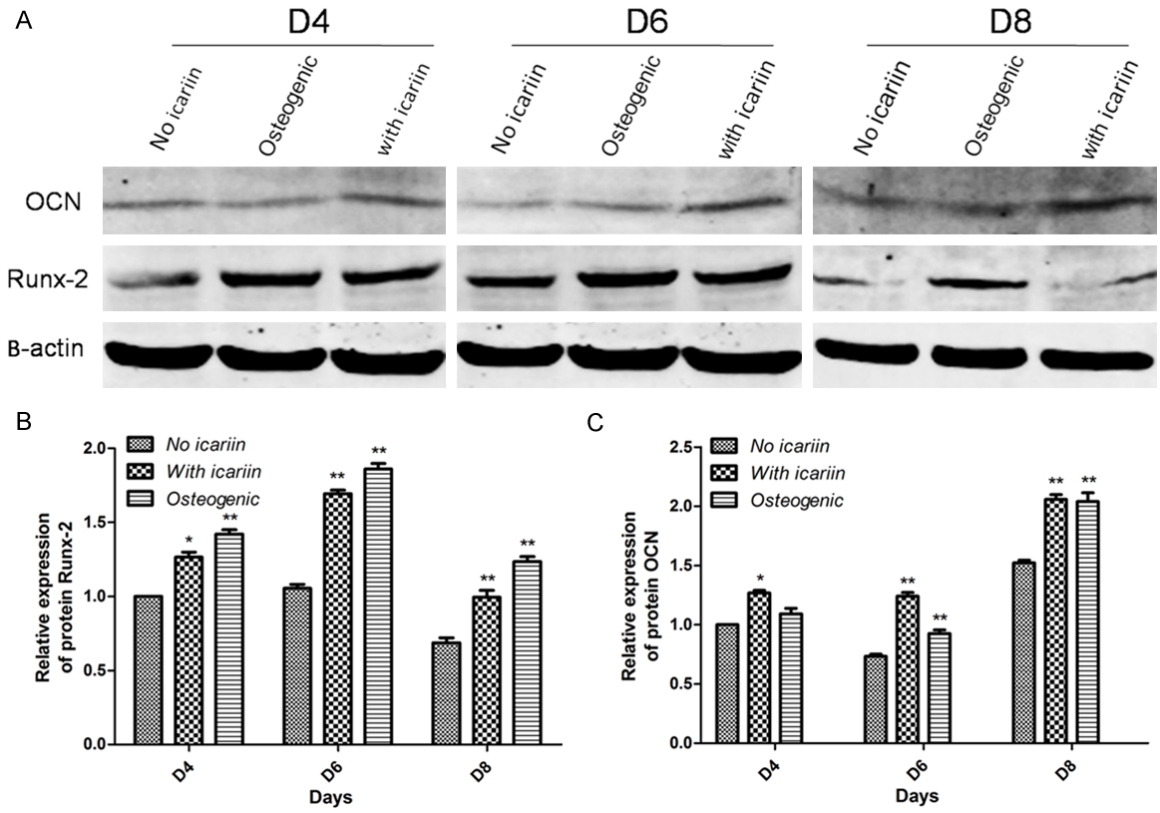

Figure 7. Expression of osteogenesis-related proteins in BMSCs after treatment with 10^{-6} M icariin for 4, 6, and 8 days, assessed by Western blot (A). Graphs depicting fold changes of Runx2 (B) and osteocalcin (OCN) (C). Complete

medium without icariin served as negative control, while the osteogenic induction group served as positive control. Data are mean ± standard deviation (SD). *P<0.05 and **P<0.01 indicate statistically significant differences between the experimental (treated with 10^{-6} M icariin) and control (no icariin) groups at the same time point.

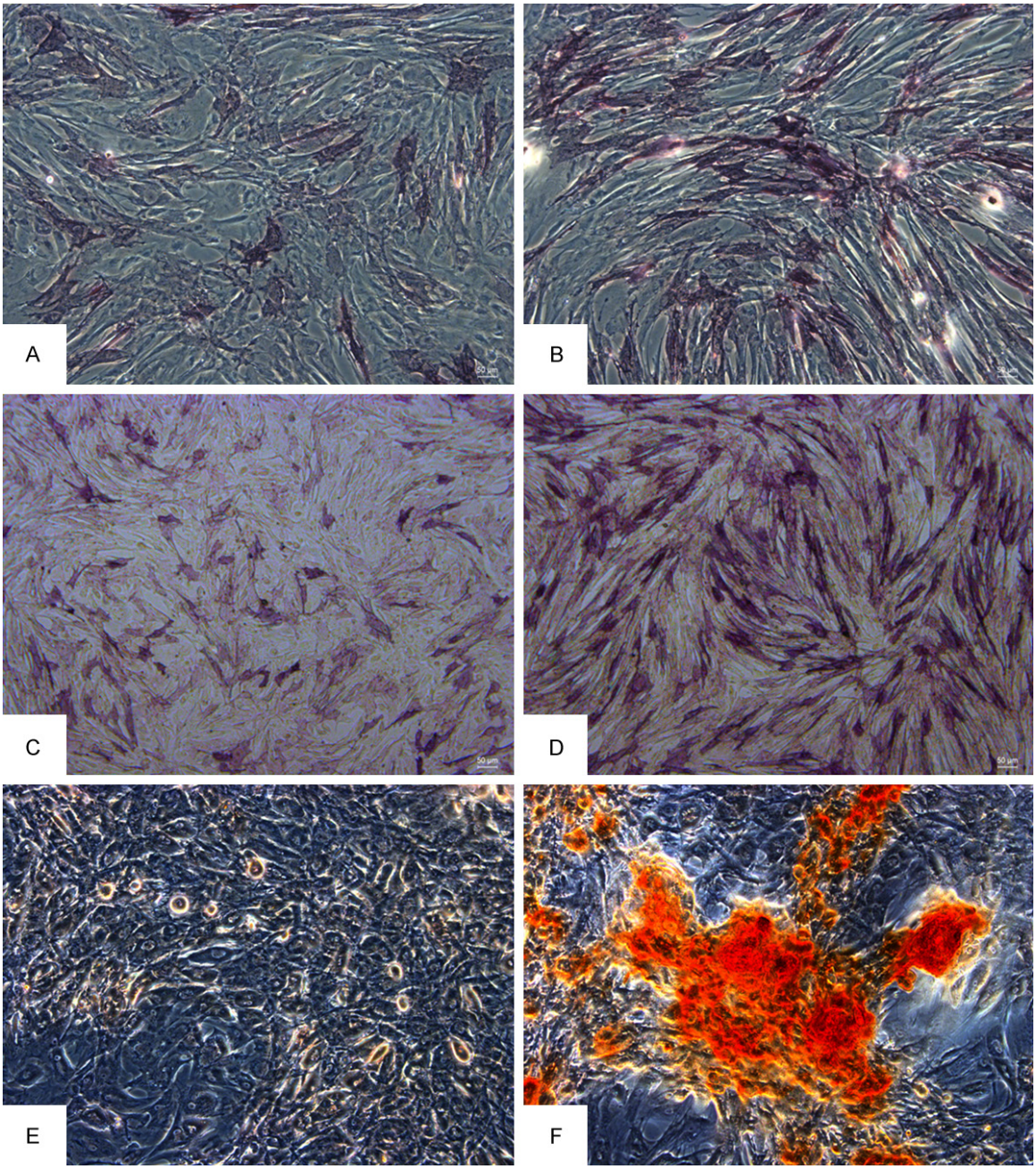

Figure 8. Alkaline phosphatase (ALP) and alizarin red (ARS) staining. (A-D) ALP staining of BMSCs at 7 days. ALP expression levels were significantly enhanced with BMSCs treated with 10^{-6} M/L icariin (B, D) compared with complete medium (A, C). Magnification ×100 (C, D), ×200 (A, B); (E, F) Alizarin red staining of BMSCs at 21 days. Calcium nodules were formed only with BMSCs cultured in 10^{-6} M/L icariin loaded medium (F) compared with complete medium (E). Magnification ×100.

although not statistically significant. According to the results of cell proliferation and alkaline phosphatase activity assays, 1×10^{-6} mol/l ICA was selected for subsequent experiments.

ICA up-regulates osteogenic related genes in BMSCs

To assess the gene expression levels of osteogenic genes in BMSCs after 10^{-6} mol/l ICA induction, bFGF, OCN, osterix, and Runx-2 were quantified by real-time fluorescence quantitative PCR at 3, 5, and 7 days, respectively (**Figure 6**). At 5 days after ICA induction, the expression levels of the osteogenic related genes bFGF ,OCN, osterix, and Runx-2 were overtly increased compared with those of the negative control (NC) group, with statistically significant differences ($P<0.01$). The expression levels of bFGF in the ICA group were higher than that of the NC group at 7 days ($P<0.01$).

ICA up-regulates osteogenic related proteins in BMSCs

To assess the effect of ICA on osteogenic related proteins in BMSCs, the latter were stimulated with 10^{-6} mol/l ICA for 4, 6, and 8 days, respectively. The results showed that protein expression levels of Runx-2 were increased by 1.5 to 2 fold in the 10^{-6} mol/l ICA group compared to the negative control group (**Figure 7B**). Similar findings were obtained for OCN (**Figure 7C**). Specifically, at 8 days, the Runx-2 protein was less expressed that at 6 days; at 8 days, the late protein OCN showed higher amounts than that at 4, 6 days. These results were in accordance with qPCR data, and indicated that 10^{-6} mol/l ICA could upregulate osteogenic proteins in BMSCs *in vitro*.

ICA enhances early osteogenic differentiation and final mineralization in BMSCs

BMSCs were plated into 6-well plates (10^5 cells/well). Seven days after treatment with 10^{-6} mol/l ICA, expression levels of the early osteogenic differentiation marker ALP were significantly enhanced compared with control cells (**Figure 8A-D**). Furthermore, Alizarin red staining at 21 days revealed a significant increase in calcium deposition after ICA treatment (**Figure 8E**, **8F**). These results are consistent with gene and protein expression patterns of osteogenic markers in BMSCs. Taken together, these findings indicated that 10^{-6} mol/l ICA promoted osteogenic activity in BMSCs.

Discussion

BMSCs are widely used in bone tissue engineering; under appropriate conditions, they can be stimulated into osteoblasts, fat cells, and cartilage cells, by cell differentiation. However, BMSCs only constitute 0.01% of bone marrow cells [26-28]; bone marrow adherent cells in addition to BMSCs, are also mixed with fibroblasts and various stromal cells. This study used the whole bone marrow culture method; cell isolation involves a great deal of suspension blood cells and various adherent cells of the bone marrow. According to growth characteristics of bone marrow cells, we adopted different ways to exclude them one type at the time, to obtain high purity BMSCs. Non-adherent blood cells could be removed by changing the medium. For adherent cells, removal was performed by adjusting the trypsin digestion time (0.25% of pancreatic enzyme for 2 min); macrophages and fibroblasts attached tightly to the plate cannot be digested in 2 min, and could be cultured. Despite the International Association of Cell Therapy appraisal standard for human BMSCs [29], appraisal standards remain to be explored for other species. Currently, whether a group of cells are mesenchymal stem cells can only be based on cell morphology, multi-directional differentiation properties, and cell biology indexes [30]. We found that dog BMSCs (dBMSCs) were adherent and fusiform, with typical fibroblast morphology; after induction into osteogenesis and adipocytes, calcium nodules and lipid droplets can be observed, respectively. This means dBMSCs have the characteristics of stem cells, and can be used for bone tissue engineering.

A suitable concentration of traditional Chinese medicine in dBMSCs should be determined. In this study, 10^{-9}~10^{-6} mol/L ICA significantly promoted dBMSC proliferation and alkaline phosphatase activity. We integrated cell proliferation and osteogenesis differentiation, and selected the optimal concentration to be 10^{-6} mol/L ICA. ALP, which is necessary in osteoblasts for calcium salt deposition, is an important component involved in bone metabolism; it is also the most commonly used biochemical marker of bone formation [31], and the essential condition for mineralization. By hydrolyzing organophosphates, ALP releases inorganic phosphorus, promoting calcium phosphate precipitation. Meanwhile, ALP degrades calcification inhibitors, initiates and furthers the calcification process, and participates in the synthesis of inorganic calcium phosphate [32]. ALP is the specific enzyme in bone formation [33], and

its activity largely reflects the state and degree of osteoblast differentiation and maturation. Indeed, high ALP activity indicates the differentiation of BMSCs to osteoblasts [34]. In this study, the ICA group showed significantly higher ALP activity compared with the control group.

Osteoblasts synthesize and secrete bFGF, OCN, Osterix and Runx-2 [35]. Real-time PCR was performed for detecting the gene expression levels of bFGF, OCN, Osterix and Runx-2, while Western blot was carried out to assess OPN, OCN and Runx-2 for protein expression, to confirm the effect of ICA on BMSC osteogenesis. As shown above, 10^{-6} mol/L ICA significantly upregulated osteogenesis related factors in dBMSCs, both at the gene and protein levels.

Osteoblasts cultured *in vitro* can continuously deposit calcium salts and form calcium nodules [36], the formation of mineralized nodules is a unique sign to osteoblasts. We used 10^{-6} mol/L ICA to treat dBMSCs for 21 d, and white nodules formed at the bottom of the dish were visible to the naked eyes. After alizarin red staining, much more calcium nodules were observed in the experimental group compared with the control group, consistent with ALP data in the early stage of osteogenesis. These findings confirmed that osteogenesis induced in cultured dBMSCs results in the production of osteoblast calcium nodules, demonstrating that ICA dose dependently promotes osteogenetic differentiation of dBMSCs.

In summary, ICA improves BMSC proliferation and alkaline phosphatase activity, and induces the differentiation of beagle's BMSCs, increasing the expression of osteogenesis related factors at the gene and protein levels and promoting osteoblast mineralization. These findings suggested that ICA can promote the osteogenetic differentiation of dBMSCs. In the current study, the optimal concentration of ICA was 10^{-6} mol/L.

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Disclosure of conflict of interest

None.

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