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Original Research Article

Osteogenic and Angiogenic Differentiation of Bone Mesenchymal Stem Cells Induced by Different Concentrations of Icariin

Pharmacognosy Magazine
22(4) 1301–1309, 2026
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DOI: 10.1177/09731296251388230
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Abstract

Background: Bone defects resulting from trauma or lesions are common orthopedic conditions that challenge bone regeneration. Bone mesenchymal stem cells (BMSCs) have garnered attention due to their osteogenic potential. Icariin has shown promise in promoting osteogenic and angiogenic differentiation in BMSCs through pathways such as BMP, MAPK, and Wnt/β-catenin. However, the optimal concentration of icariin and its dual osteogenic and angiogenic roles remain unclear.
Objectives: This study was designed to explore the osteogenic and angiogenic differentiation of BMSCs induced by icariin at different concentrations.
Materials and Methods: Primary human BMSCs were cultured with varying concentrations of icariin (0–40 μM) for 21 days. Cell Counting Kit-8 (CCK-8) assay assessed cell proliferation. Phase-contrast microscopy, quantitative real-time polymerase chain reaction (qRT-PCR), and Western blotting evaluated osteogenic markers (Runx2, VEGF). Angiogenic differentiation was analyzed through cell morphology, transmission electron microscopy, and the expression of VEGF and CD31.
Results and Conclusion: Proliferation assays indicated that 1 μM icariin significantly enhanced BMSC viability compared to the control group ($p < .05$). Osteogenic differentiation, marked by increased Runx2 and VEGF expression, was most pronounced at 1 μM. Elevated VEGF and CD31 expression and the presence of Weibel–Palade bodies in transmission electron microscopy images confirmed angiogenic differentiation. High concentrations (20–40 μM) exhibited cytotoxicity, reducing proliferation and differentiation. Collectively, icariin at 1 μM optimally induces osteogenic and angiogenic differentiation in BMSCs, making it a potential therapeutic agent for bone defect repair. These findings offer insights into the dual biological function of icariin, paving the way for advanced bone regeneration strategies. Future studies should focus on validating these effects in preclinical animal models.

Keywords

Icariin, Weibel–Palade bodies, angiogenic differentiation, osteogenic markers

Received 14 January 2025; **revised** 08 June 2025; **accepted** 18 September 2025

Introduction

Bone defects or non-union because of trauma or lesions are prevalent in orthopedic diseases, often resulting in reduced bone formation and bone mass. Bone mesenchymal stem cells (BMSCs) have garnered significant attention as a promising therapeutic approach for these conditions due to their ability to differentiate into osteoblasts and promote bone tissue regeneration (Arthur & Gronthos, 2020; Zhu et al., 2024). These adult stem cells, originating from the mesoderm, possess the capacity to differentiate into various cell lineages, including osteoblasts, chondrocytes, and endothelial cells (Khorasani et al., 2021; Purwaningrum et al., 2021). This unique ability has positioned BMSCs as a vital resource for bone defect repair and regeneration.

Icariin, a prenylated flavonoid glycoside derived from *Epimedium brevicornum*, is a well-known bioactive constituent of traditional Chinese medicine. Because of its diphenylpropane (C6-C3-C6) structure, icariin acts as a plant-derived phytoestrogen and has been extensively used in managing

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postmenopausal osteoporosis, fractures, and bone metabolic disorders (Ding et al., 2025; Wang et al., 2018; Yuan et al., 2025). It mimics estrogenic effects by binding to estrogen receptors, especially ER-β, without eliciting the adverse effects associated with hormone replacement therapy. At the molecular level, icariin exerts multifaceted actions on bone regeneration by activating key osteogenic signaling pathways. These include the BMP-2/Smad1/5/8 axis (Huang et al., 2020), the Wnt/β-catenin pathway (Yang et al., 2019), and MAPK-dependent signaling cascades (Wu et al., 2015), all of which culminate in upregulation of transcription factors, such as Runx2 and Osterix. These transcription factors play essential roles in the initiation and maturation of osteoblastic differentiation. Additionally, icariin suppresses adipogenic lineage commitment by downregulating PPARγ expression, preserving the osteogenic potential of BMSCs (Jin et al., 2025).

Beyond osteogenesis, icariin promotes angiogenesis, a critical component of bone repair, by upregulating angiogenic markers such as VEGF and bFGF. These effects are believed to be mediated through PI3K/Akt and ERK/MAPK signaling pathways, contributing to endothelial cell proliferation, migration, and tubular structure formation (Yang et al., 2019). Recent evidence has shown that icariin-loaded biomaterials or scaffolds significantly enhance neovascularization and bone integration in tissue-engineered constructs (Jin et al., 2025). Although the biological potential of icariin has been widely documented, the optimal concentration range for maximizing its dual osteogenic and angiogenic effects remains unclear. Previous studies have reported efficacious results across a broad range of concentrations, from nanomolar to low micromolar levels (Wang et al., 2018). Such inconsistency underscores the need for a systematic investigation into dose-response relationships.

Furthermore, while the *in vitro* osteogenic and angiogenic activities of icariin have been studied independently, fewer studies have explored its concurrent induction of both processes in BMSCs. Given the interdependence of angiogenesis and osteogenesis during bone formation, elucidating this dual functionality is critical for improving regenerative outcomes. This study aims to determine the optimal concentration range of icariin for inducing osteogenic differentiation in BMSCs and assess its ability to promote angiogenic differentiation concurrently. By systematically analyzing these dual effects across multiple concentrations, this work seeks to clarify the bipotential mechanism of icariin and its implications for bone defect repair and tissue engineering.

Materials and Methods

Cell Culture

Primary human BMSCs were procured from iCell Bioscience Inc., Shanghai, and identified by immunofluorescence. Cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The third passage of cells was used for all experiments. Cells were seeded at appropriate densities depending on the assay: 5×10^4 cells/well in 96-well plates for the Cell Counting Kit-8 (CCK-8) assay and 2×10^5 cells/well in 6-well plates for differentiation studies.

CCK-8 Assay

To evaluate the proliferation activity of BMSCs, third-passage cells were treated with complete media containing icariin at concentrations of 0 μM, 0.1 μM, 1 μM, 10 μM, 20 μM, and 40 μM. The CCK-8 assay (Apexbio, product no. K1018) was used to measure cell proliferation according to the manufacturer's instructions. Optical density (OD) values were measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (ELX808; BioTek Instruments, Inc.) at 0 h, 24 h, 48 h, and 72 h. Each group included three biological replicates, and each measurement was performed in technical triplicate.

Osteogenic Differentiation Detection

BMSCs were cultured in icariin-containing media with varying concentrations (0 μM, 0.1 μM, 1 μM, 10 μM, 20 μM, 40 μM) for 21 days. On the 21st day, phase-contrast microscopy was used to observe cell morphology, while quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were performed to analyze osteogenic differentiation markers and protein expression. Morphological evaluations were performed on days 1, 7, 14, and 21.

Angiogenic Differentiation Detection Test

The angiogenic differentiation detection test evaluated the effects of icariin and growth factors on BMSCs. Four groups were tested: (a) the blank group (DMEM/F12 + 10% FBS, 10 ng/mL bFGF, 10 ng/mL hVEGF, and 1% double-antibody), (b) the control group (blank group medium + 1 μM icariin), (c) the induction group (blank group medium + VEGF and bFGF), and (d) the experimental group (induction group medium + 1 μM icariin). BMSCs were cultured for 21 days, followed by microscopic observation of cell morphology, ultrastructure analysis using transmission electron microscopy (TEM), and additional tests such as qPCR and Western blotting for angiogenic markers. Phase-contrast and TEM imaging were performed on day 21.

Phase-contrast Microscopy for Cell Morphology Visualization

To assess the effects of icariin on BMSC morphology, cells were cultured at different concentrations of icariin and

observed at specified time points. Cell morphology was imaged using a phase-contrast microscope (Olympus IX73). Morphological parameters such as cell size, shape, and density were monitored. Images were captured at 200× magnification, and visual changes were documented for comparison among treatment groups. Although this study employed qualitative assessment, future work will include quantitative image analysis.

qRT-PCR

For gene expression analysis, total RNA was extracted from BMSCs using the Ultrapure RNA Kit (Cwbio, product no. CW0581), and complementary DNA (cDNA) was synthesized using ReverTra Ace-α (TOYOBO, product no. FSK-100) according to the manufacturer's protocols. qRT-PCR was then performed to assess the expression levels of RUNX2, VEGF, and CD31 using gene-specific primers. GAPDH served as the internal control. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and all reactions were conducted in triplicate to ensure reproducibility. The primer sequences used were as follows:

RUNX2: Forward 5′-CCTGAACTCTGCACCAAGTC-3′, Reverse 5′-GGCTGCTGTTTGTGCTGTTG-3′
VEGF: Forward 5′-AGGGCAGAATCATCACGAAGT-3′, Reverse 5′-AGGGTCTCGATTGGATGGCA-3′
CD31: Forward 5′-TTTGACAAAGTGTCCTGCAGAG-3′, Reverse 5′-AGCAGGTCAGTGAACAGGGA-3′
GAPDH:Forward5′-ACAACTTTGGTATCGTGGAAGG-3′, Reverse 5′-GCCATCACGCCACAGTTTC-3′

TEM

The ultrastructure of BMSCs was observed using TEM to assess angiogenic differentiation. Cells from different groups were digested, centrifuged, and fixed with 1% osmium tetroxide for 1.5 h at room temperature. After fixation, samples were dehydrated through an ethanol gradient, embedded in epoxy resin, and sectioned using an ultramicrotome. Ultrathin sections of 80 nm were stained with uranyl acetate and lead citrate and visualized using a TEM (model: Hitachi HT7700) at 80 kV. Structural changes, including the presence of Weibel–Palade (W–P) bodies, were analyzed.

Western Blotting

Western blotting was conducted to analyze protein expression levels of RUNX2 and VEGF during osteogenic differentiation and VEGF and CD31 during angiogenic differentiation. Total protein was extracted using RIPA lysis buffer (Solarbio, product no. R0020-100 mL) supplemented with PMSF (Solarbio, product no. P0100). Protein concentrations were determined using a BCA kit (Solarbio, product no. PC0020). Primary antibodies used were anti-human RUNX2 (Abcam, ab236639), anti-human VEGFA (Abcam, ab214424), and anti-human CD31 (CST, 77699S). Secondary antibody used was HRP-labeled goat anti-rabbit IgG (Abcam, ab205718). Bands were visualized using ECL Plus fluorescence detection reagent (Solarbio, PE0010-A/B), and band intensity was quantified using ImageJ software.

Statistical Analysis

All experiments were performed in triplicate. Results are expressed as mean ± standard deviation. Statistical comparisons were conducted using one-way analysis of variance (ANOVA) in GraphPad Prism software (version 9). *Post hoc* comparisons were performed using Tukey's test. A $p < .05$ was considered statistically significant.

Results

Effects of Icariin on BMSC Proliferation

BMSCs treated with various concentrations of icariin exhibited dose-dependent changes in proliferation, as assessed by CCK-8 assay. Significant increases in OD, indicative of enhanced cell viability, were observed at 24 h, 48 h, and 72 h in the 0.1 μM and 1 μM icariin groups compared to the control group ($p < .05$). Notably, at 72 h, the 1 μM icariin group displayed the highest proliferation rate, with an OD value approximately 1.3 times greater than that of the control group (Figure 1A). Conversely, higher icariin concentrations (20 μM and 40 μM) resulted in decreased proliferation, suggesting potential cytotoxic effects at these levels. Phase-contrast microscopy confirmed increased cell confluence in all treatment groups at 72 h; however, no marked morphological changes were observed under qualitative evaluation (Figure 1B). Quantitative analysis of cell morphology (e.g., cell area, shape factor) was not performed in this study but is planned for future investigations.

Effects of Icariin on Osteogenic Differentiation of BMSCs

After a 21-day culture period with various icariin concentrations, BMSCs in the 0.1 μM and 1 μM groups exhibited significantly increased cell density. Phase-contrast microscopy revealed enlarged cell bodies with distinct and dense nucleoli in these groups, while the 20 μM and 40 μM groups showed lower cell density (Figure 2A). Runx2 protein expression, as detected by Western blotting, was highest in the 1 μM group and significantly higher than in the control group ($p < .05$; Figure 2B). qRT-PCR analysis showed a similar pattern, with the highest relative Runx2 mRNA expression observed in the 1 μM group, followed by a decline at higher concentrations (Figure 2C). The 20 μM and 40 μM groups exhibited expression levels comparable to the control ($p > .05$). VEGF expression was also evaluated in the context of osteogenic differentiation. Western

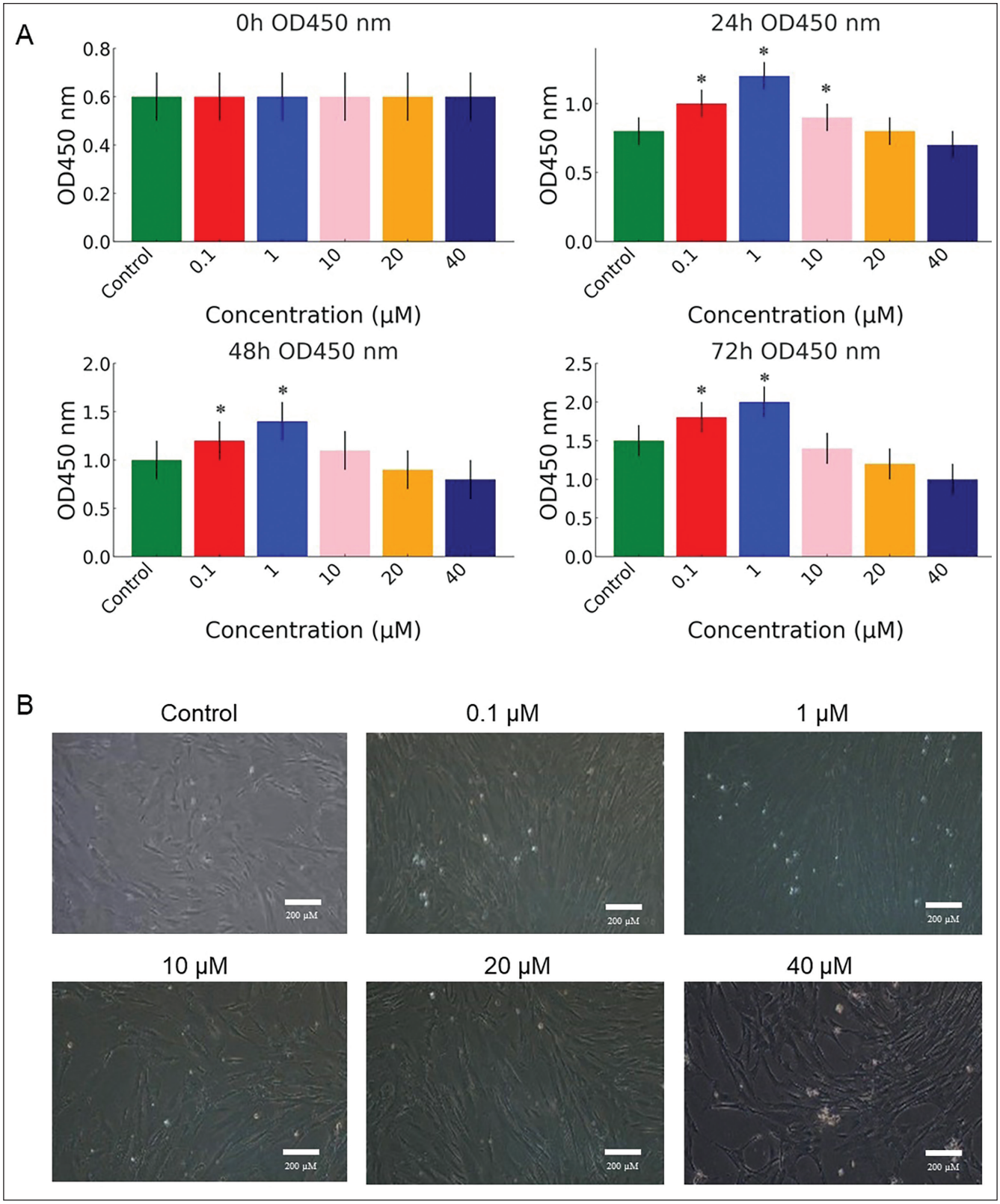


Figure 1. Effects of Icariin on Bone Mesenchymal Stem Cell (BMSC) Proliferation (A) Cell Counting Kit-8 (CCK-8) Assay Showing the Effects of Icariin on Cell Proliferation at Different Concentrations and Time Points (B) Phase-contrast Microscopy Showing Effect of Different Concentrations of Icariin at 72 h of Incubation.

Note: Data are presented as mean ± SD (*$p < .05$).

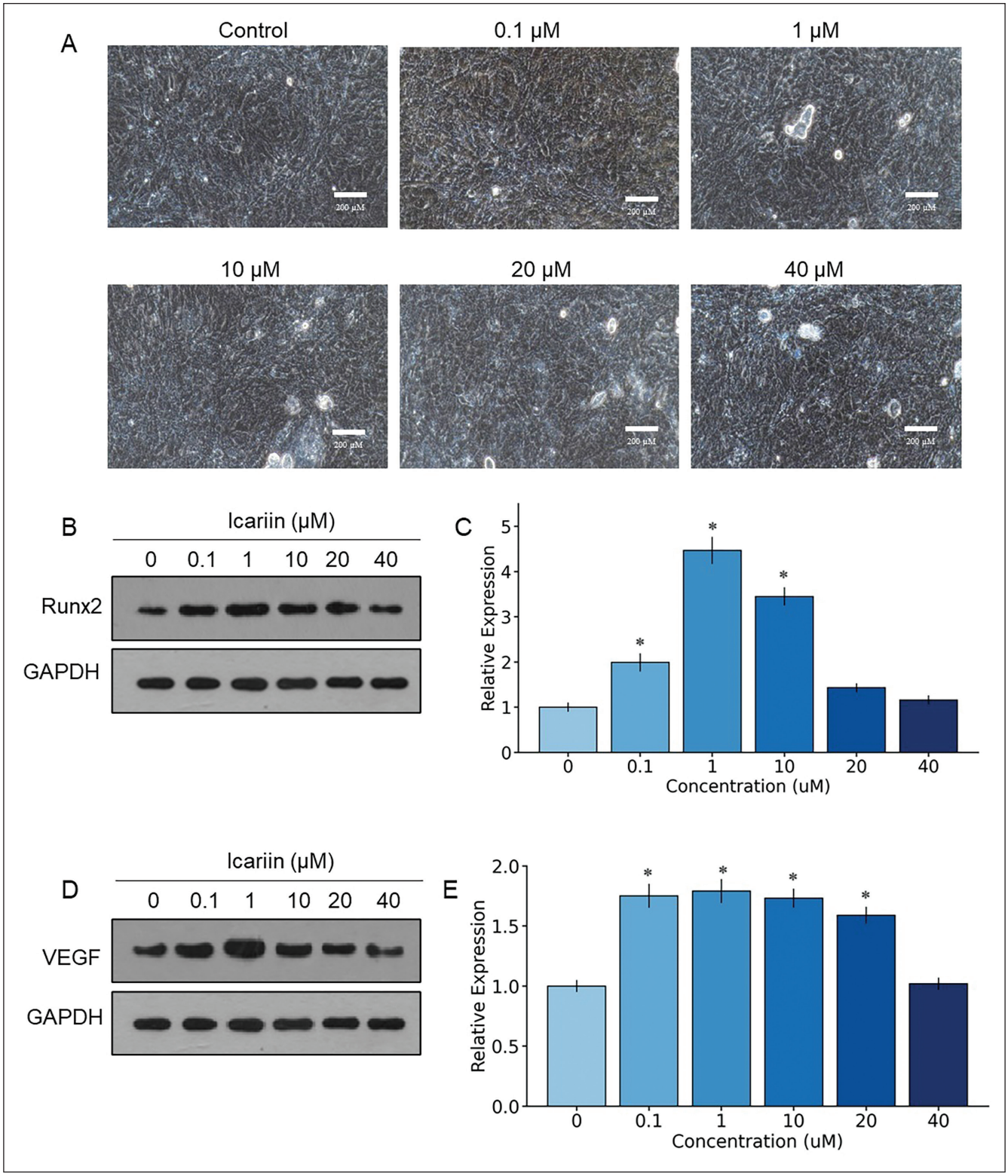


Figure 2. Effects of Icariin on Osteogenic Differentiation of Bone Mesenchymal Stem Cells (BMSCs) (A) Phase-contrast Microscopy Showing Effects of Different Concentrations of Icariin on the Morphology of BMSCs After 21 Days of Incubation (B) Western Blots Showing Expression of Runx2 in BMSCs After 21 Days of Incubation (C) Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Showing mRNA Levels of Runx2 in BMSCs After 21 Days of Incubation (D) Western Blots Showing Expression of VEGF in BMSCs After 21 Days of Incubation (E) qRT-PCR Showing mRNA Levels of VEGF in BMCs After 21 Days of Incubation.

Note: Data are presented as mean ± SD (*$p < .05$).

blotting indicated that VEGF protein levels were significantly upregulated in the 1 μM group compared to the control (Figure 2D), and qRT-PCR confirmed the highest VEGF mRNA expression at 1 μM and the lowest at 40 μM (Figure 2E). Although VEGF is a classical angiogenic factor, its expression during osteogenic differentiation reflects its role in promoting endochondral ossification and matrix remodeling.

Effects of Icariin on Angiogenic Differentiation of BMSCs

Given the strong osteogenic and proliferative response to 1 μM icariin, this concentration was selected to evaluate its angiogenic potential. Cell morphology under phase-contrast microscopy at 200× magnification demonstrated increased cell size and prominent nucleoli in the control, induction, and experimental groups following 21 days of culture (Figure 3A). Transmission electron microscopy revealed W–P bodies in the induction and experimental groups, indicating endothelial-like differentiation. These bodies appeared elongated or circular, with tightly packed tubular structures arranged in a swirl and surrounded by an electron-dense matrix (Figure 3B). The presence of W–P bodies supports the angiogenic activation of BMSCs under the influence of icariin. Western blotting showed elevated VEGF and CD31 protein levels across all groups, and the experimental group (VEGF + bFGF + 1 μM icariin) exhibited the highest expression (Figure 4A). CD31 protein expression in the experimental group reached

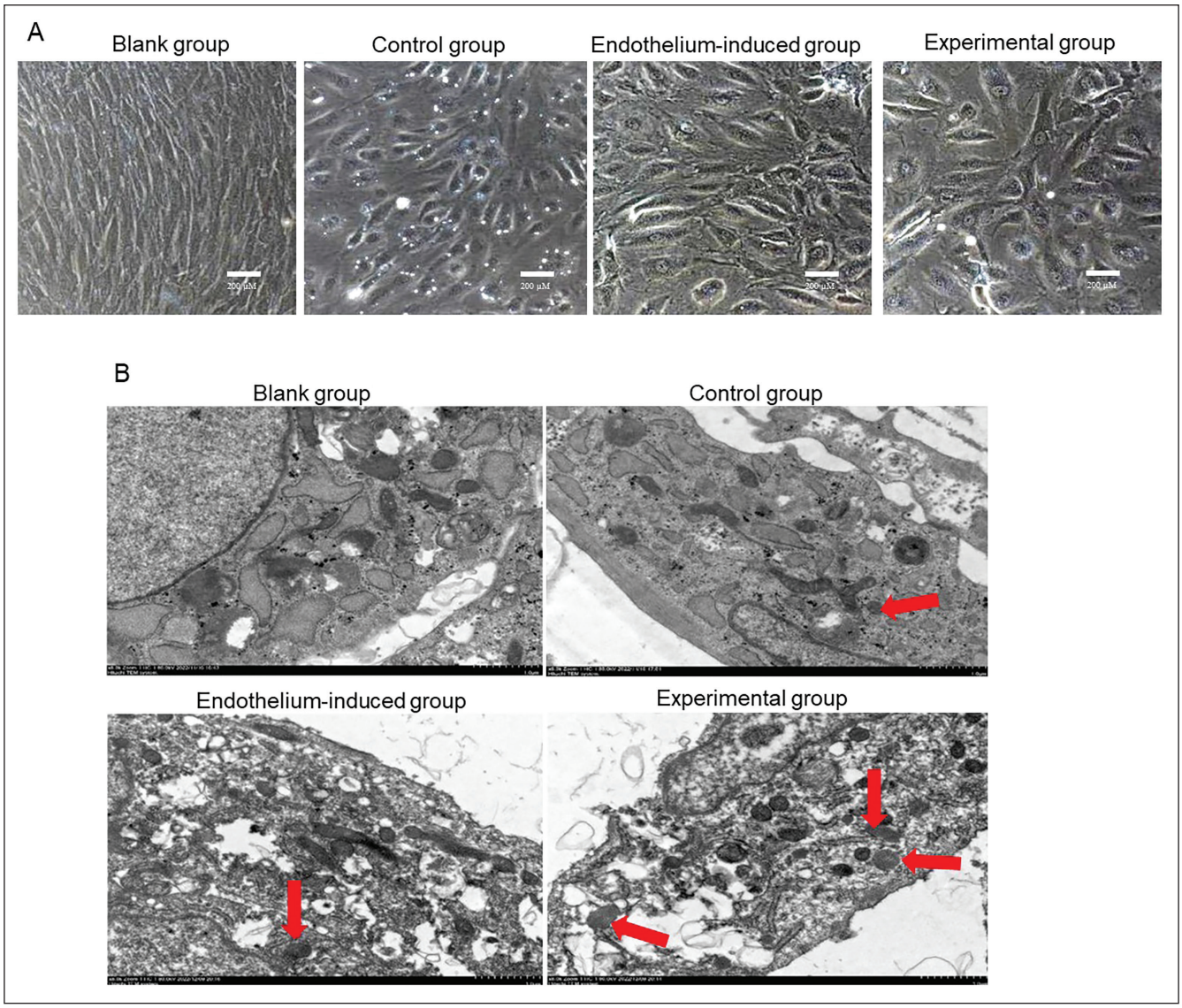


Figure 3. Effects of Icariin on Angiogenic Differentiation of Bone Mesenchymal Stem Cells (BMSCs) (A) Phase-contrast Microscopy Showing Effects of Icariin on the Morphology of Indicated Study Group BMSCs (B) Transmission Electron Microscopy (TEM) Images Showing Effects of Icariin on the Morphology of Indicated Study Group BMSCs. Red Arrows Depict Weibel–Palade Bodies. Experiments were Performed in Triplicate.

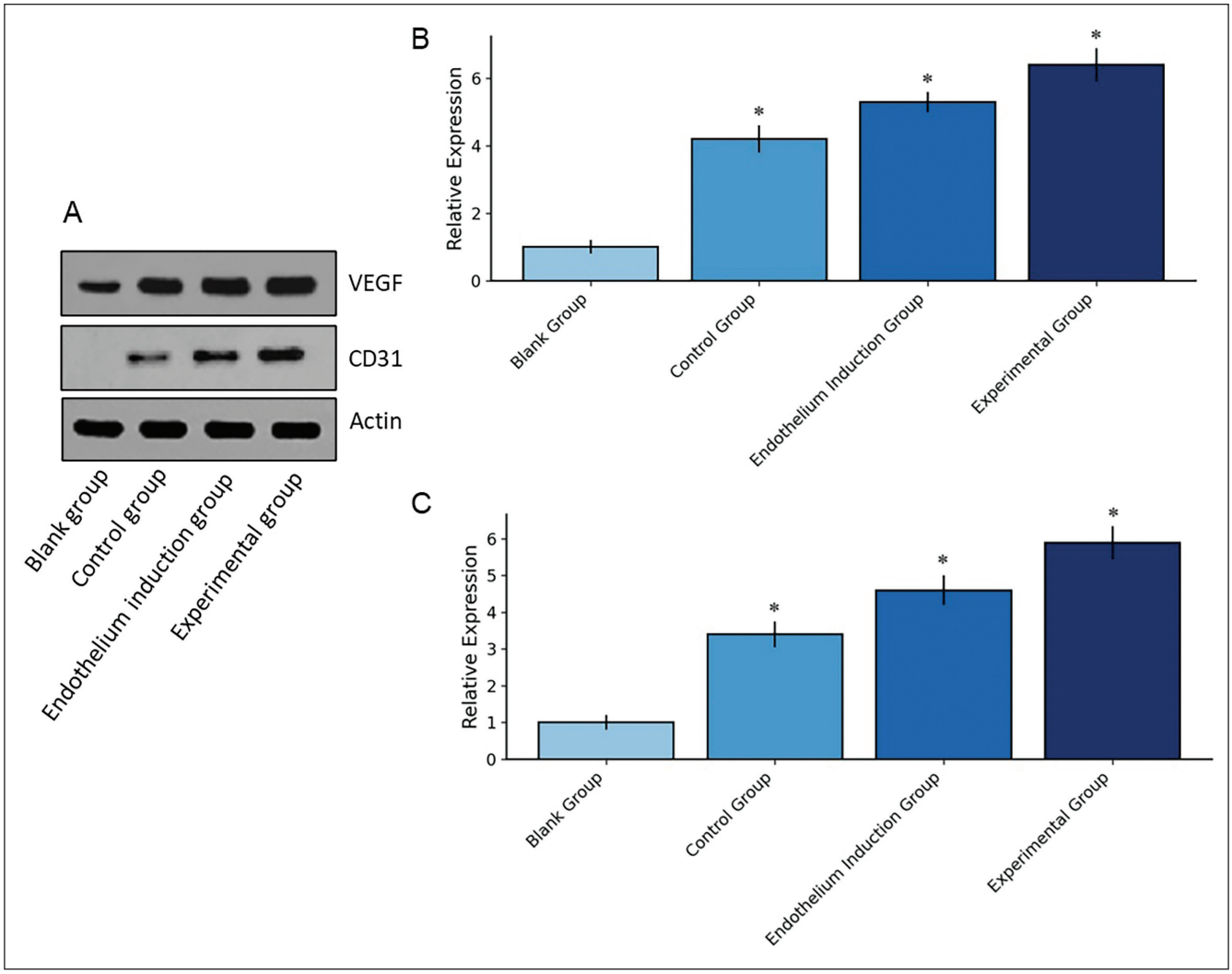


Figure 4. Effects of Icariin on Angiogenic markers in Bone Mesenchymal Stem Cells (BMSCs) (A) Western Blots Showing Expression of VEGF and CD31 in Indicated Study Group BMSCs. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Analysis Showing Expression of (B) VEGF, and (C) CD31 in Indicated Study Group BMSCs.

Note: Data are presented as mean ± SD (*$p < .05$).

0.85 AU, significantly higher than the induction and control groups ($p < .05$). The blank group showed no detectable CD31 protein. The qRT-PCR analysis revealed a similar trend in gene expression: both VEGF and CD31 mRNA levels were highest in the experimental group, confirming the enhancement of angiogenic differentiation at both transcript and protein levels (Figures 4B and 4C).

Discussion

Icariin, a bioactive flavonoid glycoside extracted from *Herba Epimedii*, has been widely regarded as a phytoestrogen due to its estrogen-like chemical structure and ability to bind estrogen receptors, thereby mimicking estrogenic activity and contributing to bone metabolism (Wang et al., 2018; Xiao et al., 2016). This structural similarity has led to its therapeutic application in osteoporosis and fracture healing. Previous studies have shown that icariin enhances the proliferation and osteogenic differentiation of BMSCs through several signaling pathways, including ERK, p38 MAPK, BMP/Smad, and Wnt/β-catenin (Huang et al., 2020; Wu et al., 2015; Yang et al., 2019). Icariin has also been reported to inhibit osteoclast differentiation and modulate the osteogenic–osteoclastic coupling necessary for bone remodeling (Xie et al., 2019).

Microscopic observations of this study revealed that after treating BMSCs with different concentrations of icariin for 72 h, the cells treated with icariin showed higher confluence compared to the control group. Furthermore, CCK-8 assay results demonstrated significantly higher cell viability in icariin-treated groups, indicating a proliferation-promoting effect of icariin. Notably, after 21 days of culture, cell density and morphology improved significantly in the 0.1 μM, 1 μM, and

10 μM groups, while higher concentrations (20 μM and 40 μM) led to sparser cell distribution. These findings emphasize that icariin supports BMSC proliferation at lower concentrations (0.1–10 μM) but may exhibit cytotoxic or differentiation–inhibitory effects at higher doses. This biphasic response is supported by Fan et al. (2011) who reported enhanced hBMSC proliferation and osteogenesis with icariin concentrations ranging from 0.001 to 1 μM but reduced viability at >10 μM. Similarly, Wu et al. (2015) found no cytotoxicity at concentrations up to 10 μM but significant toxicity beyond this threshold. In line with these findings, our study identifies 1 μM as the optimal concentration for dual promotion of BMSC proliferation and differentiation.

Runx2 and VEGF are critical markers of osteogenic differentiation and angiogenesis, respectively. Runx2 regulates osteoblast lineage commitment, while VEGF supports matrix remodeling and vascular invasion during ossification (Liu & Olsen, 2014; Schipani et al., 2009). Our results showed that BMSCs treated with 1 μM icariin displayed the highest mRNA and protein levels of Runx2 and VEGF, indicating maximal osteogenic activity. These findings are consistent with studies showing that icariin upregulates Runx2 via BMP/Smad signaling and promotes angiogenesis by activating VEGF expression (Huang et al., 2020; Yang et al., 2019; Zhao et al., 2008). Importantly, VEGF's dual role in both angiogenesis and osteogenesis reflects the interdependence of vascular and bone tissue development. We clarify that in our study, VEGF expression was interpreted in both osteogenic and angiogenic contexts, consistent with its established function in coupling bone formation and neovascularization (Diomede et al., 2020; Kanczler & Oreffo, 2008). To further validate angiogenic differentiation, we assessed the expression of CD31, a well-established endothelial marker, and performed ultrastructural analysis via TEM. W–P bodies, unique to endothelial cells, were observed in icariin-treated groups, thereby confirming endothelial lineage commitment. This finding adds a novel morphological dimension to icariin's angiogenic potential, supporting earlier studies that relied solely on molecular assays (Zhu et al., 2024). Moreover, previous work has shown that icariin can enhance mitochondrial activity and ATP production in BMSCs, thus predisposing them toward angiogenic differentiation (Huang et al., 2016). Our Western blotting and qRT-PCR analyses confirmed elevated expression of both VEGF and CD31 in icariin-treated cells, especially in the experimental group containing growth factors and 1 μM icariin.

Despite interesting results, there are some limitations of our study, which include the exclusive use of *in vitro* assays and lack of *in vivo* studies, absence of mineralization-specific tests (such as ALP or Alizarin Red staining), and lack of apoptotic marker assessment. Furthermore, quantitative morphological assessments were not performed, which could have added strength to our conclusions. Future studies should focus on *in vivo* validation using murine calvarial or femoral defect models and explore 3D co-culture systems to better simulate the bone microenvironment. Moreover, the development of icariin-loaded scaffolds may provide synergistic benefits in promoting both osteogenesis and angiogenesis (Jin et al., 2025).

Conclusion

In conclusion, our study demonstrates that even at a low concentration (1 μM) icariin can simultaneously induce osteogenic and angiogenic differentiation of BMSCs, highlighting its potential as a therapeutic agent for bone regeneration. This concentration significantly upregulated Runx2 and VEGF for osteogenesis and CD31 for angiogenesis, confirming its dual action. These findings deepen our understanding of icariin's mechanisms and its role in promoting BMSC differentiation, thereby providing a foundation for translational strategies in regenerative medicine. However, as the present study was conducted *in vitro*, future *in vivo* studies are warranted to validate these effects in animal models and thus explore their clinical applicability, including its potential use in icariin-loaded scaffolds or combinatorial therapies for bone defect repair.

Abbreviations

BMSCs: Bone mesenchymal stem cells; BMP: Bone morphogenetic protein; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval and Informed Consent

Not applicable.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The study was supported by funding from Suzhou Science and Technology Bureau Science and Technology Development (SKJYD2021189) and The People's hospital of suzhou new district Scientific Innovation Fund General Project (SGY2021B02).

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