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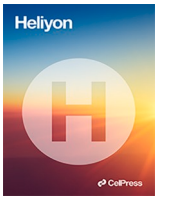

Icariin negatively regulated lipopolysaccharide-induced inflammation and ameliorated the odontogenic activity of human dental pulp cells *in vitro*

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ABSTRACT

Alleviating inflammation and promoting dentine regeneration is critical for the healing of pulpitis. In this study, we investigated the anti-inflammatory, angiogenesis and odontogenesis function of icariin on Human dental pulp cells (HDPCs) under inflammatory state. Furthermore, the underlying mechanisms was also evaluated. Icariin attenuated the LPS-induced pro-inflammatory marker expression, such as interleukin-1β (IL-1β), IL-6 and IL-8. The immunoblotting and immunofluorescence staining results showed that icariin suppressed the inflammatory responses mediated by the protein kinase B (Akt) and nuclear factor kappa-B (NF-κB) signaling cascades. Additionally, icariin also upregulated the expression of odontogenic and angiogenic genes and proteins (namely dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), anti-collagen I (COL-I), and vascular endothelial growth factor (VEGF) and fibroblast growth factor-1 (FGF-1)), alkaline phosphatase activity, and calcium nodule deposition in LPS-exposed HDPCs. In a word, our findings indicated that icariin attenuated pulp inflammation and promoted odontogenic and angiogenic differentiation in the inflammatory state. Icariin may be a promising vital pulp therapy agent for the regenerative treatment of the inflamed dental pulp.

1. Introduction

The dental pulp is a loose mesenchymal tissue rich in nerve and blood vessels, which keeps the organic components of the surrounding mineralized tissues supplied with moisture and nutrients. Unlike other tissues, the pulp occupies a non-expandable chamber of enamel, dentin, and cementum [1]. Once the microorganisms and their toxins invade into the pulp, the solid dentin walls encourage a higher interstitial fluid pressure, increased vessel permeability, stagnation of blood circulation, and micro-abscesses formation [2,3]. If neglected, ischemia and necrosis of the pulp may result [4].

Dental pulpitis is a typical pulpal inflammatory response caused by oral pathogenic bacteria that reach the pulp by penetrating the dentinal tubules [5]. Gram-negative bacteria commonly exist in caries and pulpitis [6]. Lipopolysaccharide (LPS) is a primary

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component of the Gram-negative bacteria membrane, and is responsible for pulp infection. Hence, many research models have used Gram-negative bacteria (for the LPS) to induce reliable and reproducible pulp inflammatory reactions to mimic caries-related infection [7,8]. During pulpal inflammation development, LPS activates macrophages to secrete proinflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1(IL-1) and further induces the release of mediators and chemokines, such as IL-6, IL-8 and prostaglandin E_2(PGE_2), which amplifies the inflammatory responses including pulp cell apoptosis and tissue necrosis [9–15].

Inflammation is a double-edged sword for pulp repair/regeneration [16,17]. The slow progression of mild inflammation is instrumental to pulpal repair and tertiary dentin formation. In contrast, chronic severe inflammation causes cell apoptosis or necrosis, leading to pulpal destruction [18,19]. To reduce microorganism invasions and control inflammation, it is vital to accelerate healing and elevate timely self-repair [20]. Pulp exposure can cause bacterial contamination of pulpal tissue, even leading to lose pulp viability [21]. Conventional pulp capping materials such as calcium hydroxide ($Ca(OH)_2$)-, mineral trioxide aggregate (MTA)-, calcium silicate-, and adhesive-based materials have drawbacks in clinical application, such as high cost, cytotoxicity, long setting time, and its application must be in an area free from any infections [22,23]. Therefore, there is an urgent need to develop some alternative alleviating inflammation and facilitating dentine regeneration drugs with higher efficacies and lower costs.

Icariin (molecular formula: $C_{33}H_{40}O_{15}$), is a major bioactive compound found in the Epimedium herb, and is a natural multi-functional prenylated flavanol [24,25]which offers broad pharmacological functions including anti-inflammatory actions [26], antioxidant effects [27], and osteogenic differentiation regulation [28,29] both in vivo and *in vitro*. Icariin is also beneficial for alleviating pyroptosis-induced osteoarthritis and reducing collagen formation in chondrocytes [30]. The results from Xu et al.'s study also supported icariin as the potential target drug for oestrogen-deficiency–induced alveolar bone loss [31]. Previously, we demonstrated that icariin modulates odontogenic differentiation in human dental pulp cells (HDPCs) by regulating the MAPK signaling pathway [21]. However, icariin's effect on the regulation of inflamed HDPCs remains unclear. Hence, this study aimed to investigate whether icariin has anti-inflammatory and ameliorating odontogenic activities in pulpal inflammation. Furthermore, the underlying molecular mechanisms of icariin in suppressing inflammation of HDPCs were explored.

2. Materials and methods

2.1. Primary culture of HDPCs

The Ethics Committee of the Chonnam National University Dental Hospital approved all the study protocols (IRB No: CNUDH 2022-006). HDPCs were collected from extracted clinically healthy third molars after informed consent. Then, the cells were maintained in α-minimum essential medium (α-MEM, Gibco Invitrogen, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco Invitrogen) at 37 °C in a humidified incubator with 5 % CO_2. When the primary HDPCs reached 80 % confluency, they were subcultured. To prevent altered cell behaviours caused by prolonged culturing, HDPCs at passages 3–5 were used for the following *in vitro* experiments.

2.2. Cell treatment

After confluence, the cells were incubated with icariin (Sigma Proproof, Dutendorfer, Germany) for 1 h following treatment with LPS (1 μg/mL, *Escherichia coli*, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. For experiments related to the mineralization potential, HDPCs were cultured in an odontogenic induction medium (OM) containing 50 μg/mL of ascorbic acid (Sigma-Aldrich) and 10 mmol/L of β-glycerophosphate (Stata Cruz, Dallas, TX, USA).

2.3. Cell viability assay

The Ez-Cytox Enhance Cytotoxicity Assay kit (Dogen, Seoul, Korea) was used to analyse the cell viability of the HDPCs. Briefly, 5×10^3 cells were exposed to 0.1 μg/mL, 0.5 μg/mL and 1 μg/mL of LPS for 24 h or increasing concentrations of icariin (2.5 μM, 5 μM, 10 μM or 20 μM) and 1 μg/mL of LPS for 1, 3, 5 and 7 days, respectively. A growth medium was used as the control. After treatment, 10μl of Ez-Cytox Enhance solution was added to each well, and continuously incubated for 1 h at 37 °C. The absorbance at a wavelength of 450 nm was measured using a spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.4. Enzyme-linked immunosorbent assays

Following the indicated treatment, all supernatants were collected and stored in aliquots at −80 °C until analysis. The amount of prostaglandins 2 (PGE_2), interleukin-6 (IL-6) and interleukin-8 (IL-8) secreted into the media was measured using a commercially available ELISA kit (R & D Systems, Minneapolis, MN, USA) and a microplate reader.

2.5. Alkaline phosphatase (ALP) and alizarin red S (ARS) staining assay

After the indicated treatment, HDPCs were cultured in OM media for 10 days. The cells were fixed for 10 min with 4 % paraformaldehyde. Subsequently, the cells were stained with 250 μL of 1-step NBT/BCIP reagent (Thermo Fisher Scientific, Rockford, IL, USA) for 10 min under gentle agitation according to the manufacturer's instructions. To observe calcium deposition in the extracellular matrix, ARS staining was performed after the HDPCs have been maintained in the OM medium for 14 days. The cells were then fixed

and stained with 2 % ARS (Lifeline Cell Technology, Frederick, MD, USA) at room temperature for 20 min. The mineral deposition-positive area was subjected to visual inspection and imaging. To quantify the calcium concentration, the red dye was dissolved in 10 % cetylpyridinium chloride (CPC, PH = 7.0) and optical densities were measured at 540 nm using the spectrophotometer.

2.6. Total RNA extraction and real-time PCR analysis

After the cells were cultured, the total RNA of the HDPCs was harvested using Trizol. The extracted 2 μg RNA was reverse transcribed in a final volume of 15.25 μL using the AccessQuickTM RT-PCR kit (Promega, Madison, WI, USA) for cDNA synthesis. RT-PCR amplifications were performed using the QuantiNova™ SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). The thermal cycling was set as follows:95 °C for 5min and 45 cycles at 95 °C for 5s, 60 °C for 10s, 72 °C for 20 s. The $2^{-\triangle\triangle CT}$ method was employed to calculate the relative gene expression values. The primer sequences used for amplification are listed in Table 1.

2.7. Protein isolation and western blot analysis

HDPCs were seeded into 60 mm dishes with a density of 3×10^5 cells/well. At the indicated time points, the cultured HDPCs were harvested and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, USA) with phenylmethanesulfonyl fluoride (Sigma-Aldrich). The extracted proteins were loaded for electrophoresis and transferred to polyvinylidene difluoride membranes, and then, incubated with the appropriate primary antibodies including MyD88 (Santa Cruz), vascular cell adhesion molecules (VCAM), intercellular adhesion molecules (ICAM), vascular endothelial growth factor (VEGF), anti-collagen I (COL-I) (Abcam, Cambridge, UK), cyclooxygenase 2 (Cox 2), NF-κB p65, phospho–NF–κB p65, IκB-α, phospho-IκB-α (Cell Signaling Technology), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1) and fibroblast growth factor-1 (FGF-1) (Thermo Fisher Scientific), at 4 °C overnight. β-actin (Cell Signaling Technology) and used as the internal control. Subsequently, the membranes were developed with antirabbit horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the Western blot detection system (Ez-capture; Atto, Tokyo, Japan) was used to capture the signals.

2.8. Immunofluorescence staining

To detect intracellular p65 localization. HDPCs were seeded into 48-well plates, fixed with 4 % paraformaldehyde for 30min at 25 °C, and permeabilized with 0.1 % Triton X-100 in PBS for 15min. The cells were then incubated with 1 % BSA for 1 h and with NF-κB p65 (1:300; Abcam) antibody overnight at 4 °C. Then, the cells were incubated in secondary antibodies solution-Alexa Fluor 488 conjugate (1:500 cell signaling Technology). The nuclei were stained with diluted DAPI (Molecular Probes) and fluorescence was observed using a confocal laser scanning microscope (Lionheart™ FX, Winooski, VT, USA).

2.9. Statistical analysis

Each independent experiment was repeated in triplicate. GraphPad Prism 8 (GraphPad software) was used for all statistical analyses. Quantitative data are expressed as mean ± standard deviation (SD). The significant difference between the control and experimental groups was analysed using one-way analysis of variance (AVOVA). $P < 0.05$ was considered statistically significant.

3. Result

3.1. The effect of icariin on cytotoxicity in LPS-induced HDPCs

To confirm the applicable LPS concentration for the *in vitro* inflammation model, HDPCs were treated with 0.1 μg/mL, 0.5 μg/mL

Table 1
Primer sequences.

Genes	Forward primer 5′-3′	Reverse primer 5′-3′
IL-1β	TCA ATA TTA GAG TCT CAA CCC CCA TTC TCT TTC GTT CCC GGT GG	
IL-6	TAA TGG GCA TTC CTT CTT CT TGT CCT AAC GCT CAT ACT TTT	
IL-8	TTT CTG TTA AAT CTG GCA ACC CTA GT ATA AAG GAG AAA CCA AGG CAC AGT	
VCAM	TTC CCT AGA GAT CCA GAA ATC GAG GTT GCA GCT TAC AGT GAC AGA GC	
ICAM	CTC CAA TGT GCC AGG CTT G CAG TGG GAA AGT GCC ATC CT	
DMP 1	TGG TCC CAG CAG TGA GTC CA TGT GTG CGA GCT GTC CTC CT	
DSPP	GGG AAT ATT GAG GGC TGG AA TCA TTG TGA CCT GCA TCG CC	
OCN	CTC ACA CTC CTC GCC GTA TT GCT CCC AGC CAT TGA TAC AG	
VEGF	GAG GAG CAG TTA CGG TCT GTG TCC TTT CCT TAG CTG ACA CTT GT	
FGF-1	GAC GAC TCT ATG CTT CGG AGC AGG CGT ACT AGA CAC CGT CC	
GAPDH	CAT CAC CAT CTT CCA GGA G AGG CTG TTG TCA TAC TTC TC	

Interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), intercellular adhesion molecules (*ICAM*), vascular cell adhesion molecules (*VCAM*), dentin matrix protein 1 (*DMP1*), dentin sialophosphoprotein (*DSPP*), osteocalcin (*OCN*), vascular endothelial growth factor (*VEGF*), fibroblast growth factor (*FGF-1*), glyceraldehyde-phosphate dehydrogenase (*GAPDH*).

and 1 μg/mL LPS for 24 h. As shown in Fig. 1A, there was no statistical difference among the LPS groups. Moreover, an increased protein level of Cox 2 and PGE_2 release induced by 1 μg/mL LPS indicated that there was inflammation (Fig. 1B and C) (**$p < 0.01$, ***$P < 0.001$). Based on our results, the concentration of 1 μg/mL LPS was selected for subsequent experiments.

To evaluate the cytotoxicity of icariin on LPS-stimulated HDPCs, the cells were pretreated with 2.5 μM, 5 μM, 10 μM and 20 μM of icariin, and then, stimulated with 1 μg/mL of LPS simultaneously at day 1, 3, 5 and 7. The results showed that the 20 μM icariin/1 μg/mL LPS group gradually began to exert a transient suppressive influence on the cell viability rate of the HDPCs on day 5 compared with the control group (Fig. 2) (*$p < 0.05$). None of the control, LPS or icariin-treated groups (2.5 μM, 5 μM and 10 μM) showed cytotoxicity.

3.2. Anti-inflammatory properties of icariin

To determine the role of icariin on the anti-inflammatory capabilities of LPS-induced HDPCs, the cells were pretreated with icariin (5 μM and 10 μM) for 1 h, and then, stimulated with 1 μg/mL LPS for a further 24 h. Since IL-1β, IL-6, IL-8, VACM and ICAM are the major proinflammatory cytokines and intercellular adhesion molecules associated with pulpitis, we found that LPS dramatically increased the genes expression of IL-1β, IL-6, IL-8, VACM, ICAM, compared with the control group. Pretreatment with icariin demonstrated inhibitory effects in a dose-dependent manner (Fig. 3A). Similarly, icariin attenuated the VCAM, ICAM, and Cox 2 protein expression of HDPCs under the LPS-mimicked inflammatory condition (Fig. 3B). Moreover, IL-6, IL-8 proteins, and the synthesis of PGE_2 secreted into the supernatant of LPS-induced HDPCs were repressed by icariin (Fig. 3C). Collectively, these results indicated that 5 μM nd 10 μM icariin effectively suppressed LPS-induced inflammation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.3. Icariin suppressed the activation of AKT signaling in LPS-induced HDPCs

The LPS-induced AKT and NF-κB pathways are essential for the activation of inflammation [7,32]. To further observe the mechanisms through which icariin exerts its restrained influence on inflammation, we investigated the effects of icariin on the AKT and NF-κB pathways after LPS stimulation. The phosphorylation of AKT (*p*-AKT) as indicated in Fig. 4A and B, was observed by Western blot analysis. Pretreatment with 10 μM of icariin for 1 h before exposure to LPS-suppressed phosphorylation of AKT stimulated by LPS. Moreover, cells that were pretreated with 20 μM of LY294002 (AKT inhibitor, Cell Signaling Technology) had a similar influence to icariin (*$p < 0.05$).

3.4. The effect of icariin on LPS-induced HDPCs was dependent on MyD88/NF-κB signaling networks

Besides AKT signaling cascade activation, the effect of icariin on phosphorylation of NF-κB in the LPS-stimulated cells were also investigated. HDPCs was stimulated using LPS and treated with icariin for the indicated duration of time. As indicated in Fig. 4A, upstream signaling mediators MyD88 was activated within 5 min in the vehicle-treated controls. However, the MyD88 level was then subsequently suppressed after icariin treatment.

Activation of NF-κB occurred through the canonical pathway and depended on IκB protein degradation. The IκB protein was degraded when induced by proinflammatory signals, and NF-κB was released and transported to the nuclei [33]. From our immunoblotting analysis, as shown in Fig. 5A, LPS-induced phosphorylation of IκBα and NF-κB(p65) and the degradation of IκBα were significantly diminished at 30 min or 60 min after icariin treatment. Consistently, immunofluorescence staining (Fig. 5B) exhibited that p65 (green fluorescence) was clustered around the cell nuclei of untreated HDPCs, and the LPS-stimulated cells markedly promote p65 nuclear translocation. However, icariin suppressed the translocation of nuclei p65 compared to the LPS group. Taken together,

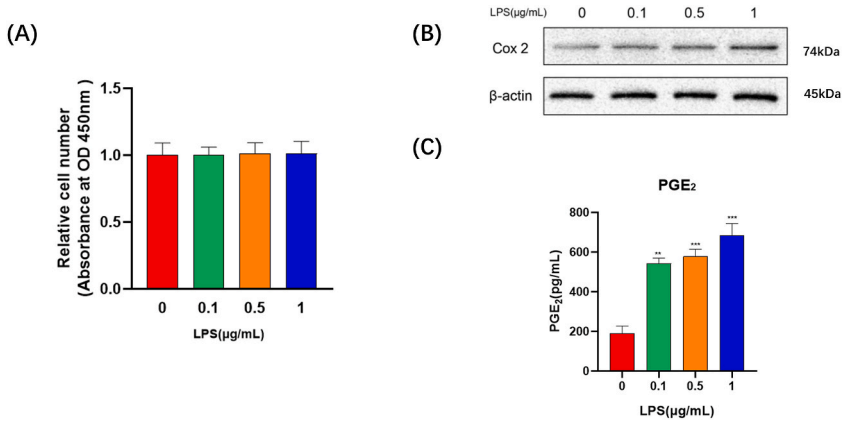

Fig. 1. The effect of LPS and icariin on HDPCs. (A) HDPCs are induced with 0, 0.1 μg/mL, 0.5 μg/mL and 1 μg/mL of LPS for 24 h. (B) The protein expression of Cox 2 is determined via immunoblotting. (C) LPS-induced PGE_2 syntheses are illustrated with enzyme-linked immunosorbent assays. (**$p < 0.01$, ***$P < 0.001$). * Represent comparisons with the control group. LPS: Lipopolysaccharide, HDPCs: human dental pulp cells, Cox 2: cyclooxygenase 2, PGE_2: prostaglandins 2.

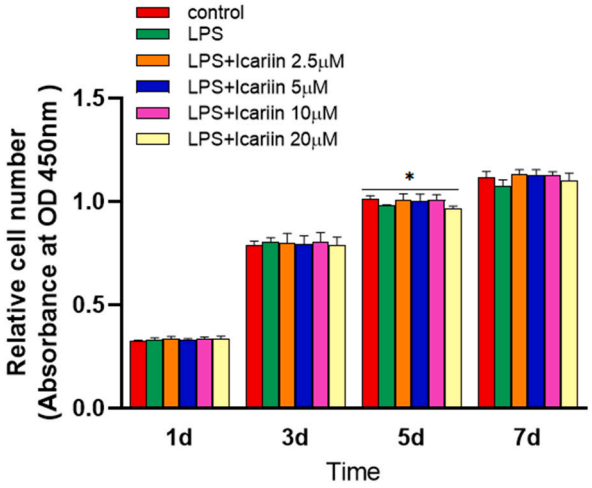

Fig. 2. The effect of icariin on LPS-stimulated HDPCs. The cells are pretreated with different concentrations of icariin (2.5 μM, 5 μM, 10 μM and 20 μM) and then, stimulated with 1 μg/mL of LPS. The cell viability is determined by the Ez-Cytox Enhance cell viability assay kit on day 1, 3, 5 and 7. (*$p < 0.05$). * Represent comparisons with the control group.

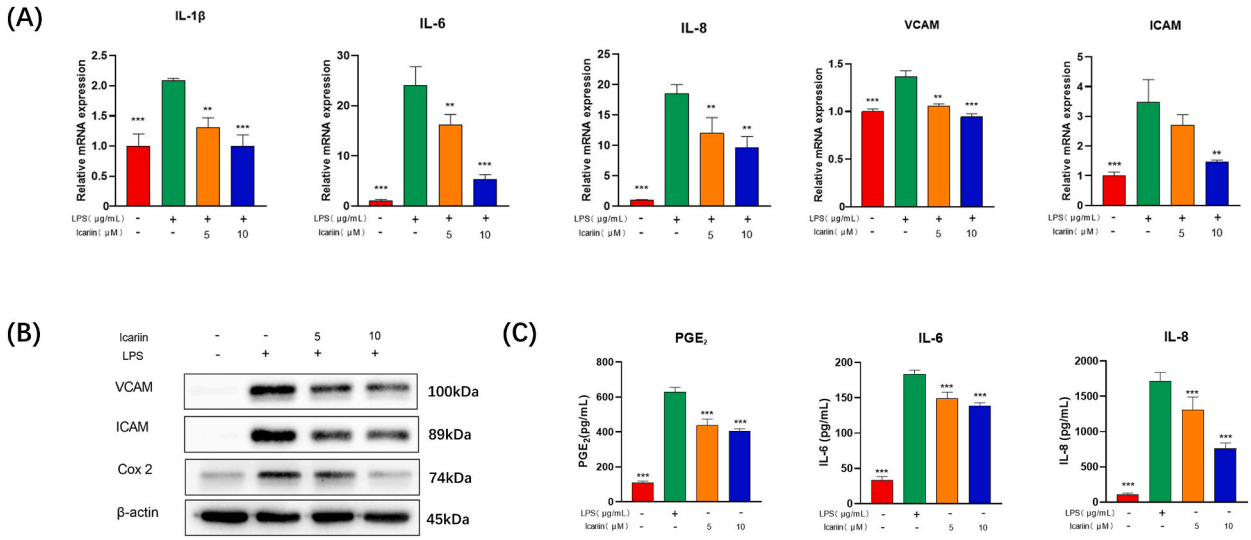

Fig. 3. The anti-inflammatory action of icariin on HDPCs. (A) LPS-treated HDPCs are cultured with 5 μM and 10 μM of icariin for 24 h. The mRNA levels of inflammatory genes, IL-1β, IL-6, IL-8, VACM and ICAM are measured by quantitative real-time PCR. (B) The protein expression of VCAM, ICAM, and Cox 2 is measured via immunoblotting. (C) The effect of different concentrations (5 μM and 10 μM) of icariin on PGE_2 release and IL-6, IL-8 proteins are detected. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). * Represent comparison with LPS-treated group. IL-6: Interleukin-6, IL-8: Interleukin-8, ICAM: Intercellular adhesion molecules, VCAM: Vascular cell adhesion molecules.

these results validate that icariin can block NF-κB signaling in LPS-induced inflammation.

3.5. Icariin ameliorated the expression of odontogenic and angiogenic genes and proteins in HDPCs under LPS-stimulated inflammation

To assess the role of icariin on the odontogenic and angiogenic capability of LPS-induced HDPCs, cells cultured in an osteogenic induction medium were treated with or without 10 μM icariin in the presence of 1 μg/mL LPS for 5 days for real-time PCR analysis and Western blot analysis. Cells were cultured in the osteogenic medium that and selected as the control group.

During cytodifferentiation analysis, the icariin-treated group was observed to enhance the protein expression levels of odontogenic (COL I, DSPP, and DMP 1) and angiogenic (VEGF, FGF-1) markers. Icariin treatment significantly strengthened the mRNA expression of *DSPP, DMP 1, OCN, VEGF* and *FGF-1* compared with the LPS-treated group on day 5(Fig. 6A). Similarly, when HDPCs were stimulated by LPS and induced inflammation, the proteins of odontogenic and angiogenic markers were restrained as compared to those in the control group. However, incubated with LPS and icariin, the cytodifferentiation abilities of HDPCs were up-regulated (Fig. 6B and C).

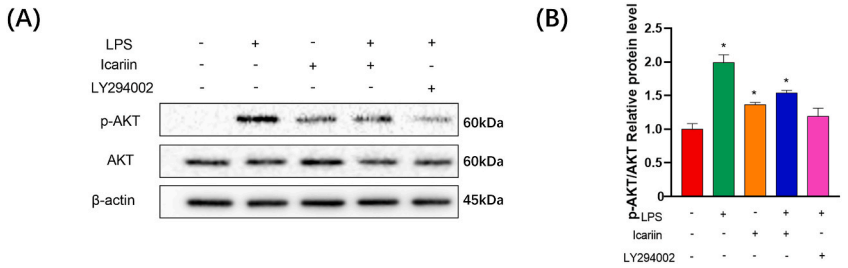

Fig. 4. The effects of icariin on the AKT pathway in LPS-induced HDPCs. (A) HDPCs are pretreated with AKT inhibitor (LY294002) followed by stimulation with LPS or icariin. The protein levels of AKT and *p*-AKT are measured by Western blot analysis. (B) The quantitative data of (A) are measured using ImageJ software. (*$p < 0.05$). * Represent comparison with the control group.

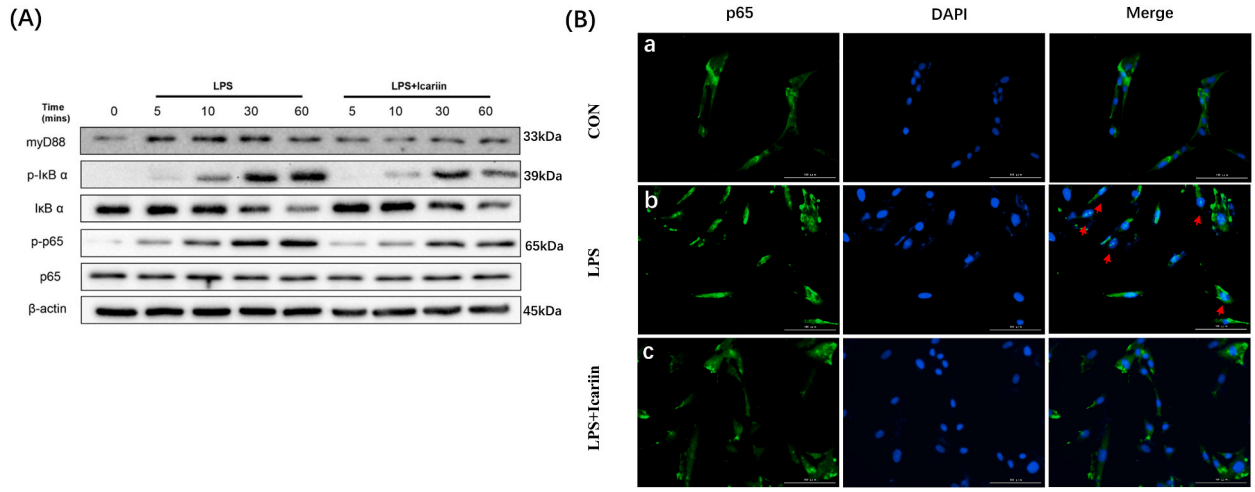

Fig. 5. Icariin attenuates inflammation via the inhibition of the MyD88/NF-κB signaling pathways. (A) HDPCs are stimulated using LPS and treated with 10 μM icariin at different time, MyD88, the phosphorylation of IκBα and p65 is detected by immunoblotting. (B) Intracellular p65 protein translocation from cytosol to nucleus are observed under immunofluorescence staining. (B-a) The green fluorescence represents NF-κB p65, which is observed in the cytoplasm around the nucleus in untreated groups. The blue fluorescence represents the nuclei stained by DAPI. (B-b) Intracellular p65 are activated and transferred to the nucleus after LPS-induced HDPCs (red arrowheads). (B-c) p65 immunolabeling in the cell nuclei is notably weak after icariin treatment.

(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). These results demonstrated that icariin usage ameliorated odontogenic and angiogenic capabilities compared with the LPS-treated group.

3.6. Icariin treatment enhanced ALP expression and resulted in mineralization nodules in LPS-induced HDPCs

To evaluate the effects of icariin on the osteogenic differentiation and mineralization of LPS-induced HDPCs, the cells were cultured with or without 10 μM icariin in the presence of 1 μg/mL of LPS. On day 10, ALP staining was restrained by LPS treatment, and icariin treatment obviously promoted this phenomenon as compared with that in the LPS group (Fig. 7A and B). Corroborate with that in the ALP results, ARS staining demonstrated that the calcium deposits were significantly attenuated in the LPS group on day 14, and the staining density improved after icariin treatment (Fig. 7C and D). (*$p < 0.05$, ***$p < 0.001$; #$p < 0.05$, ###$p < 0.001$). These findings suggested that icariin contributed to mineralization in the inflammation state.

4. Discussion

The maintenance of vital dental pulp is essential for keeping tooth proprioceptive functions, immunity, the damping property and dentinogenesis. These vital pulp functions are natural protective mechanisms that are beneficial to the long-term prognosis [34]. Dentin regeneration depends on a vital dental pulp. Advanced caries can irreversibly destroy the vital pulp by eliciting an inflammatory response that antagonizes the reparative processes [35]. Therefore, the timely reduction of inflammation and the repair of damaged dental pulp-resident cells are particularly important. In this study, we investigated whether icariin accelerated pulpal repair by adjusting to the inflammation and stimulating differentiation.

In this study, we applied 1 μg/mL of LPS to create an inflammatory microenvironment model. The successful establishment of

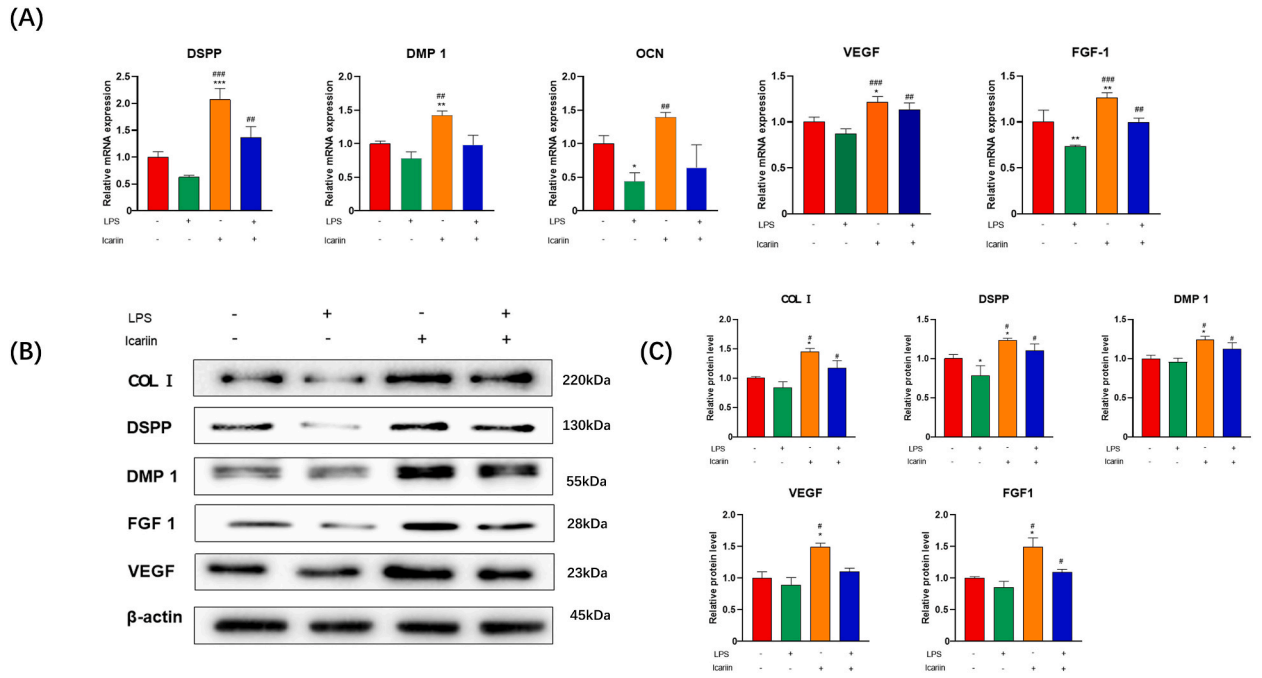

Fig. 6. Icariin's influence on the odontogenic and angiogenic differentiation of HDPCs in LPS-mimicked states. (A) The mRNA expression levels of relative genes including DSPP, DMP1, OCN, VEGF and FGF-1 are measured by quantitative real-time PCR. (B, C) The protein expression of COL I, DSPP, DMP 1, VEGF and FGF are detected by immunoblotting at 5 days. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$). * Represent comparison with the control group. # Represent comparison with the LPS group. COL-I: anti-Collagen I, DSPP: dentin sialophosphoprotein, DMP1: dentin matrix protein 1, VEGF: vascular endothelial growth factor, FGF-1: fibroblast growth factor, OCN: osteocalcin.

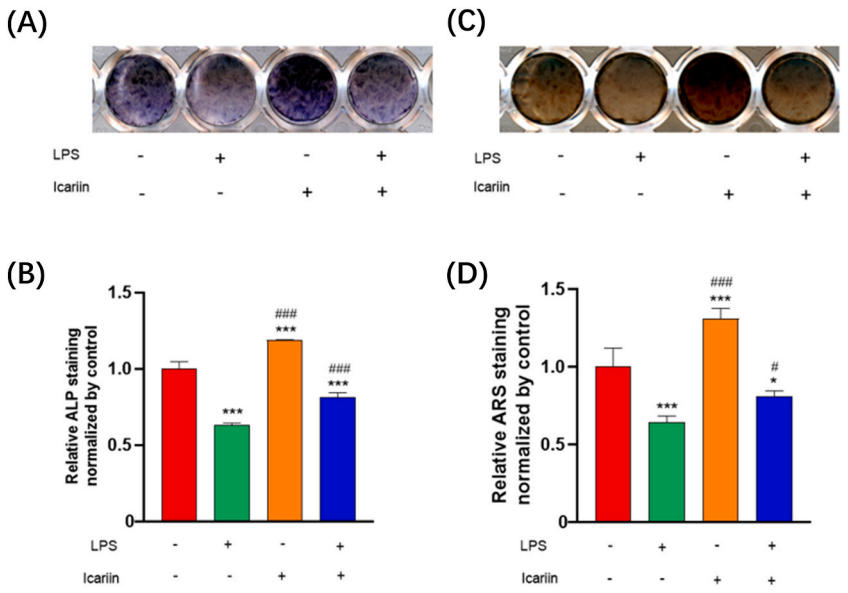

Fig. 7. The effects of icariin on the mineralization in LPS-stimulated HDPCs. (A, B) ALP staining of the HDPCs treated with or without icariin in the presence of LPS for 10 days. (C, D) ARS staining and calcium nodule quantification at day 14. ($*p < 0.05$, $***p < 0.001$; $\#p < 0.05$, $\#\#\#p < 0.001$). * Represent comparison with the control group, # Represent comparison with the LPS group, ALP: alkaline phosphatase, ARS: alizarin red S.

inflammation was confirmed by upregulating the expression of COX 2 and PGE_2. Next, we detected the biocompatibility of icariin, and the data have shown that the 20 μM/1 μg/mL LPS group has a transient suppression influence on the cell viability rate of HDPCs on day 5 compared with the control group. Therefore, we selected to investigate the effects of 5 μM and 10 μM of icariin.

PGE_2 is produced via arachidonic acid and is involved in the pathogenesis of pulpal inflammation [32,36]. Additionally, PGE_2

amplified signal transduction in sensitized nociceptors by intracellular phosphorylation, which contributes to thermal hyperalgesia, causing pain in irreversible pulpitis [37–39]. COX 2 is a rate-limiting enzyme and is involved in the excessive synthesis of PGE_2 that can lead to pathologic sequela [40,41]. In this study, the secreted PGE_2 produced a similar effect to COX 2 in HDPCs. Additionally, our data observed that icariin reduced LPS-induced pro-inflammatory cytokines including IL-1β, IL-6 and IL-8, and endothelial adhesion molecules, such as ICAM and VCAM, in a dose-dependent relationship. These findings indicated that icariin treatment may prove efficacious for pain relief by repressing inflammation.

The classical NF-κB signaling pathway was activated by a wide variety of inflammatory stimuli including LPS, and its target genes are related to the induction and development of inflammation [42]. MyD88, a toll-like receptor (TLR) 2, and the TLR4 adapter protein received stimulating signaling transfers into the cells to activate the transcription factor, NF-κB [12]. Subsequently, IκBα was activated by phosphorylation. Following IκBα degradation which cleaved the major subunits of NF-κB, the free NF-κB was translocated into the nucleus and combined with the promoter regions of the relevant genes, such as inflammatory enzymes (COX 2), chemokines and proinflammatory cytokines (IL-1, IL-6 and IL-8) [43,44]. In this study, we detected that icariin markedly attenuated the phosphorylation IκBα and NF-κB protein expression, and prompted IκBα, compared with those of the LPS-stimulated groups. Meanwhile, the expression of *p*-AKT in HDPCs increased in response to LPS and was suppressed after icariin application. Thus, this is also consistent with previous research showing that the anti-inflammatory effects of icariin on LPS-exposed cells is mediated by blocking the AKT kinase pathway, and subsequently, inactivated IκBα and NF-κB in the HDPCs [24].

Accelerating the differentiation of inflamed dental pulp cells into odontoblasts and the formation of tertiary dentin is critical for regulating pulp inflammation and facilitating pulp regeneration [20]. Therefore, we further measured the effect of icariin on repairing the HDPCs under inflammation. The differentiated HDPCs provided dentinogenesis through the formation of a collagenous web and inorganic phase mineralization to form dentin [45]. Our data demonstrated that icariin markedly upregulated the HDPCs protein expression of COL I, the major fibrous component of the pulpal extracellular matrix [46]. This indicated that icariin may facilitate the synthesis of type I collagen, which was further validated by increased ALP activities and calcium deposits on day 10 and 14 (as shown via ALP and ARS staining). VEGFs and FGFs are well-known angiogenic factors that play a key role in angiogenesis [47]. Angiogenic factors are secreted in response to injured dental pulp cells. They contribute to the formation of new capillaries and the promotion of reparative responses of the dentin-pulp complex [48]. These are also supported by icariin, which enhanced the angiogenic marker expression of VEGF and FGF-1. Based on the functional expression of osteo/odontogenic differentiation, we detected specific odontogenic markers (DSPP and DMP 1) and an osteogenic marker (OCN). In an LPS-inflamed condition, the genes and protein expression of DSPP, DMP 1 and OCN were significantly upregulated by icariin. Our results are consistent with the findings from previous studies which showed that icariin is beneficial for osteo/odontogenic differentiation of HDPCs [21].

However, there are still some limitations that need to be explored. In future experiments, we will have an in-depth study in vivo model to verify the anti-inflammation and osteo-/odontoblastic differentiation effect of icariin. And further work to evaluate the physical and mechanical properties using such an icariin-modified scaffold with HDPCs in an animal model is also necessary. In our study, we found that icariin application significantly enhanced the angiogenic marker gene and protein expressions of VEGF and FGF-1, which indicated that icariin accelerated dental pulp angiogenesis, however, the specific mechanism has not been elucidated. Future studies are needed to assess the comprehensive signaling network in an attempt to better elucidate the underlying mechanisms of icariin.

5. Conclusion

The findings of our research suggest that icariin can attenuate inflammatory cytokine release and endothelial adhesion molecule synthesis by suppressing the AKT kinase pathway and the subsequent inactivation of IκBα and NF-κB in HDPCs.

Icariin can also promote odontogenic differentiation, mineralization and angiogenesis in the inflammatory state. Thus, icariin may have the potential to be used as a vital pulp therapy agent to reduce local inflammation, alleviate pain in pulpitis and promote reactionary dentin formation.

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Data availability statement

Data associated with this study is included in this article/supplementary material/referenced in article.

CRediT authorship contribution statement

Guo Liu: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Kkot-Byeol Bae:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Ying Yang:** Writing – review & editing, Investigation, Data curation. **Bin-Na Lee:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Yun-Chan Hwang:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23282.

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