


## TRADITIONAL CHINESE MEDICINE IN ORTHOPAEDICS

## Protection of Icariin Against Hydrogen Peroxide-Induced MC3T3-E1 Cell Oxidative Damage

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**Objective:** The aim of the present study was to evaluate the potential protective mechanism of icariin against oxidative damage caused by hydrogen peroxide in MC3T3-E1 cells.

**Methods:** MC3T3-E1 cells were treated with different concentrations of icariin to explore the optimal dose of icariin. MC3T3-E1 cells were divided into groups treated with various concentrations of hydrogen peroxide ( $H_2O_2$ ; 0, 0.1, 0.2, 0.5, 1, and 2 mM) for 24 h to induce oxidative damage and cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay. Then, cells were divided into five groups: control,  $H_2O_2$  (0.2 mM), icariin (0.1  $\mu M$ ) and  $H_2O_2$  (0.2 mM) + icariin (0.1  $\mu M$ ). Cell viability was detected by CCK-8 assay. In addition, the content of glutathione and superoxide dismutase and the activity level of malondialdehyde in these treatment groups were determined. Alkaline phosphatase (ALP) and alizarin red S (ARS) staining were also performed to measure the early and late osteogenesis, respectively. Protein expression of  $\beta$ -catenin and cyclin D1 was measured by western blot assay. Then, we used an antagonist of Wnt/ $\beta$ -catenin signaling pathway (DKK-1) and western blot analysis to further explore potential mechanism.

**Results:** After 24 h of exposure to 0.2 mM  $H_2O_2$ , the viability of MC3T3-E1 cells was significantly decreased compared to that of the control cells. We first found that icariin can promote cell proliferation of MC3T3-E1 cells in a dose-dependent manner, with the dosage 0.1  $\mu M$  showing the best pro-proliferative effect. Furthermore, icariin could promote the protein expression of OSX and RUNX2. The results showed that icariin can reverse the inhibitory osteogenic effects of MC3T3-E1 caused by  $H_2O_2$ . In addition, icariin could increase the Wnt-signaling related proteins. The results showed that MC3T3-E1 cells in the  $H_2O_2$  (0.2 mM) + icariin (0.1  $\mu M$ ) + Wnt-signaling antagonist (DKK-1) group had weaker ALP and ARS staining compared with that observed in the control and  $H_2O_2$  (0.2 mM) + icariin (0.1  $\mu M$ ) groups. The ALP activity and calcium content were decreased in the 0.2 mM  $H_2O_2$  + 0.1  $\mu M$  icariin + DKK-1 group compared to that observed in the 0.2 mM  $H_2O_2$  + 0.1  $\mu M$  icariin group.

**Conclusion:** The results showed that icariin can increase the viability of MC3T3-E1 cells, reverse the oxidative stress induced by  $H_2O_2$  and protect MC3T3-E1 cells against  $H_2O_2$ -induced inhibition of osteogenic differentiation, which may occur through the Wnt/ $\beta$ -catenin signaling pathway.

**Key words:** Icariin;  $H_2O_2$ ; MC3T3-E1; Osteogenesis

### Introduction

Osteoporosis is a common metabolic bone disorder that affects approximately 75 million people all over the world<sup>1</sup>. Osteoporosis is characterized by low bone mineral density (BMD), which predisposes individuals to an increased risk of fracture<sup>2</sup>. The pathogenesis of osteoporosis is yet to be fully elucidated. Previous reports have shown that

estrogen deficiency, a lack of exercise, and oxidative stress play a central role in the onset and development of postmenopausal osteoporosis<sup>3, 4</sup>. Oxidative stress can produce excessive reactive oxygen species (ROS) and promote osteoblast damage<sup>5-7</sup>. Furthermore, ROS accelerates the apoptosis of osteoblasts, inhibiting their differentiation and impairing bone formation<sup>8</sup>. Previous studies have reported that some

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antioxidants, such as vitamin E, vitamin C, and N-acetylcysteine (NAC), can protect cells from damage induced by oxidative stress and are, therefore, considered to be alternative therapeutic agents for osteoporosis<sup>9–11</sup>. Therefore, increased oxidative stress may play an important role in bone loss and finally cause osteoporotic fractures. As a result, antioxidants may be used as a novel therapeutic approach in the prevention and treatment of osteoporosis.

Another study revealed that ROS targets the Wnt/ $\beta$ -catenin signaling pathway and hinders bone formation<sup>8</sup>. Wnt/ $\beta$ -catenin signaling plays an important role in the primary processes controlling osteogenesis, as it promotes osteoblast differentiation and mineralization<sup>12</sup>. Moreover, the inhibition of  $\beta$ -catenin by DKK-1 leads to elevated oxidative stress and reduced osteogenic capacity<sup>13</sup>. In an oxidative damage model, an aberrant WNT/ $\beta$ -catenin pathway is generally observed and leads to oxidative stress<sup>14</sup>. Taken together, these results indicate that inhibition of the Wnt/ $\beta$ -catenin signaling pathway in responding to stressors, such as oxidative stress, also suppress osteogenic differentiation.

Icariin, the major active component of *Herba Epimedii* (*Yinyanghuo* or horny goat weed), may help with postmenopausal osteoporosis (PO) treatment and prevention. Icariin has been reported to possess multiple pharmacological effects, including anti-oxidative<sup>15</sup>, anti-inflammatory<sup>16</sup>, and anti-apoptotic effects<sup>17</sup> and anti-osteoporosis activities<sup>18</sup>.

Several studies have shown that icariin has an anti-osteoporosis effect in ovariectomized rats<sup>19–21</sup>. Mok *et al.*<sup>22</sup> performed an *in vitro* study and observed that icariin exerts anabolic effects in bone, possibly by activating estrogen receptor in a ligand-independent manner. Another study revealed that icariin can protect cardiac cells from oxidative stress-induced injury through stimulation of the extracellular signal-regulated kinase (ERK) pathway<sup>23</sup>. Furthermore, icariin has osteogenic effects, primarily through activating Wnt signaling cascades<sup>24</sup>. Saud *et al.*<sup>25</sup> conducted a review to comprehensively interpret the functional role and mechanisms of icariin for cell proliferation and differentiation. They found that icariin could promote cell differentiation by targeting WNT and, finally, regulating RUNX2 expression. The protective effects and potential mechanism of icariin against oxidative damage are not yet known.

Therefore, the purpose of the present study was to examine: (i) the optimal dose of icariin for protecting against oxidative damage; (ii) whether icariin could reverse the oxidative stress induced by H<sub>2</sub>O<sub>2</sub> and protect MC3T3-E1 cells against the H<sub>2</sub>O<sub>2</sub>-induced inhibition of osteogenic differentiation; and (iii) whether icariin could activate Wnt signaling cascades to protect against oxidative damage caused by H<sub>2</sub>O<sub>2</sub>.

## Materials and Methods

### Cell Viability Assay

Cell viability was investigated using a Cell Counting kit-8 assay (CCK-8, Solarbio, Beijing, China) according to the manufacturer's instructions. We seeded 1000 cells/well in

96-well plates and then added different concentrations of H<sub>2</sub>O<sub>2</sub> (0, 0.1, 0.5, 1, and 2 mM) and icariin (0.001, 0.01, 0.1, 1, and 10  $\mu$ M). The dose of H<sub>2</sub>O<sub>2</sub> and icariin was determined based on previous studies<sup>26, 27</sup>. Then, after 24 h, 10  $\mu$ L of CCK-8 solution was added to cells in each well and incubated for 1 h. Subsequently, the absorbance was measured using an enzyme-linked immunosorbent assay reader at 450 nm.

### MC3T3-E1 Treatment with Icariin, H<sub>2</sub>O<sub>2</sub>, or their Combination

MC3T3-E1 cells were divided into four groups: a group not treated with icariin or H<sub>2</sub>O<sub>2</sub> as control, a group treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> as an oxidative stress model, a group treated with 0.1 mM icariin, and a group treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> + 0.1  $\mu$ M icariin. At 0, 12, 24, 36, and 48 h after treatment, 10  $\mu$ L of cell counting kit-8 (CCK-8) solution was added to the medium, and 1 h later, the absorbance of the samples at 450 nm was measured with a microplate reader to assess the cell viability. In addition, alkaline phosphatase (ALP) and alizarin red S (ARS) staining were performed to assess the early and late stages of osteogenic differentiation of MC3T3-E1 cells. To further elucidate the potential mechanism by which icariin regulates the osteogenesis of MC3T3-E1 cells, the Wnt inhibitor Dickkopf-1 (Dkk-1) was added, at 10  $\mu$ M, to the icariin and H<sub>2</sub>O<sub>2</sub> (0.2 mM) group.

### Culturing of MC3T3-E1 Osteoblast-Like Cells and Induction of Osteoblast Differentiation

Murine MC3T3-E1 cells were cultured in  $\alpha$ -MEM (Life Technologies) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin under an atmosphere with 5% CO<sub>2</sub> at 37°C. The medium was replaced with fresh medium every 2–3 days. When the cells reached 80% confluence, the medium was replaced with osteoblast differentiation medium ( $\alpha$ -MEM supplemented with 10% FBS, 50  $\mu$ g/mL ascorbic acid, 10 mM  $\beta$ -glycerophosphate, and 10 nM dexamethasone) for 14 days, after which the cells were used for further study. This study was approved by the ethics committee of the General Hospital of Ningxia Medical University.

### Measurement of Oxidative Stress

We measured the level of superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) to assess cellular damage using three kits obtained from Nanjing Jiancheng Biochemistry (Nanjing, Jiangsu, China) following the manufacturer's instructions. Briefly, total proteins were extracted and measured at 450 nm.

### Alkaline Phosphatase Staining and Activity Measurement

MC3T3-E1 cells were induced in differentiation medium for 7 days. For ALP staining, alkaline phosphatase staining was performed with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions<sup>28</sup>. First, we mixed 33  $\mu$ L of BCIP solution, 66  $\mu$ L of NBT solution and 10.1 mL of BCIP/

**Table 1** Gene sequences of RUNX2, OSX, and  $\beta$ -actin primers

Gene names	Forward sequence	Reverse sequence
RUNX2	5'-AAGTGCGGTGCAAACTTCT-3'	5'-TCTCGTGGGCTGCTAGTGA-3'
OSX	5'-GCCAGAAGCTGTGAAACCTC-3'	5'-GCTGCAAGCTCTCCATAACC-3'
$\beta$ -actin	5'-TCCTAGCACCATGAAGATC-3'	5'-AAACGCAGCTCAGTAACAG-3'

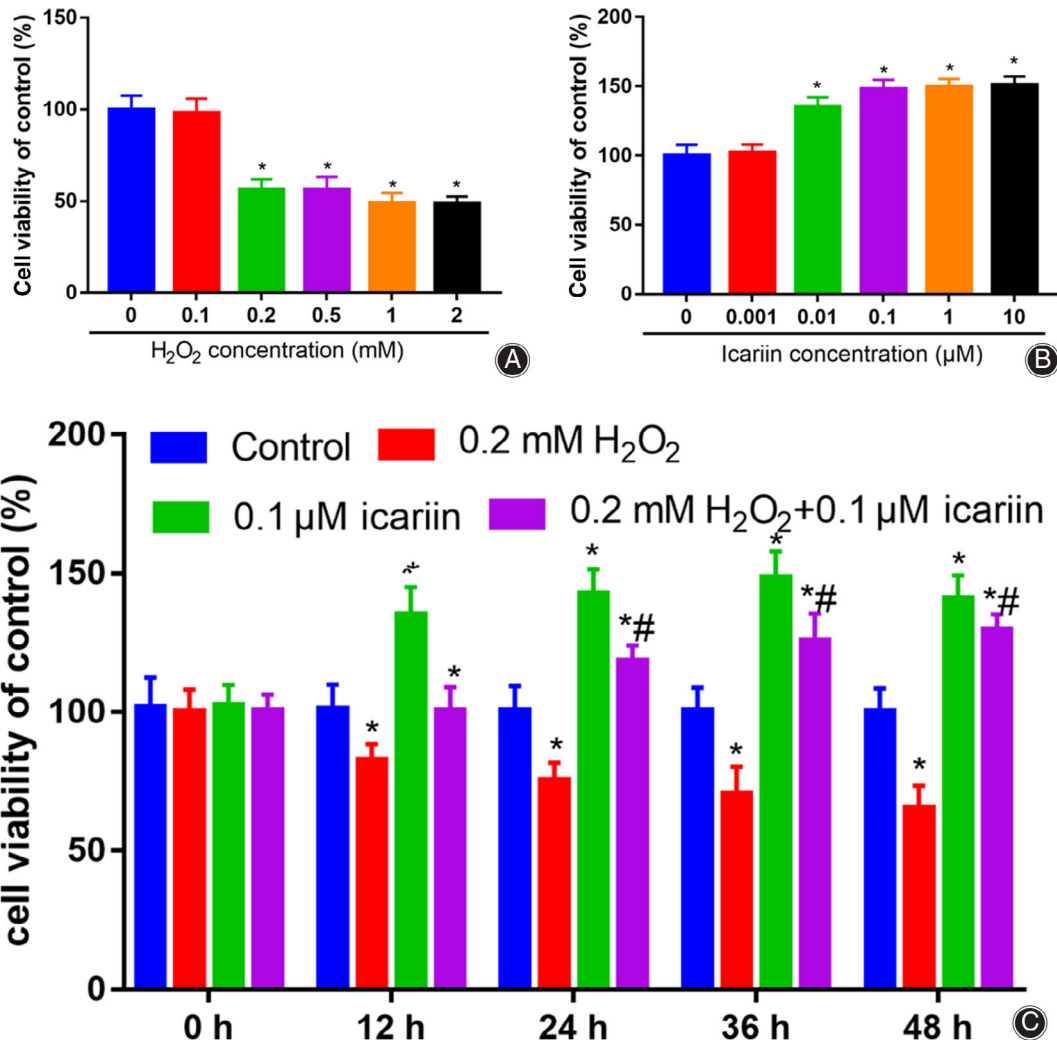
NBT dye working solution. Then, MC3T3-E1 cells were fixed with 4% paraformaldehyde and washed with PBS three times before being incubated with the mixed solutions for 10 minutes and washed with PBS to stop the reaction.

After incubating for 7 days, the MC3T3-E1 cells were harvested and washed with ice-cold PBS before being lysed by three cycles of freezing and thawing. Aliquots of supernatants from each group were used to quantitatively analyze

the ALP activity using an ALP activity kit (Nanjing Jiancheng Biological Engineering Institute, China) according to the manufacturer's instructions.

#### Alizarin Red S Staining and Calcium Assay

MC3T3-E1 cells were cultured in osteogenic induction medium before being rinsed with PBS, fixed with 4% paraformaldehyde, and stained with 0.2% alizarin red S solution



**Fig. 1** Dose-dependent effect of H<sub>2</sub>O<sub>2</sub> and icariin on MC3T3-E1 cells. (A) MC3T3-E1 cell viability when treated with different concentrations of H<sub>2</sub>O<sub>2</sub> (0.1 to 2 mM); (B) MC3T3-E1 cell viability when cocultured with different doses of icariin (0.001 to 10 µM); (C) MC3T3-E1 cell viability when cocultured with 0.2 mM H<sub>2</sub>O<sub>2</sub> and 0.1 µM icariin from 0 to 48 h. \**P* < 0.05, compared with the control group.

(pH = 8.3, Solarbio, Beijing, China) for 1 h. Orange-red staining indicated the location and intensity of calcium deposition. Calcium levels were measured with an enzyme-linked immunosorbent assay reader at 560 nm.

#### Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. A PrimeScript RT Reagent Kit (TaKaRa, Japan) was used to perform reverse transcription. Reverse-transcription polymerase chain reaction (RT-PCR) was performed using a SYBR Premix Ex Taq II kit (TaKaRa) and detected with a Roche LightCycler 480 sequence detection system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for mRNA quantitation. The gene sequences of RUNX2, OSX, and  $\beta$ -actin are presented in Table 1. The relative mRNA expression levels of RUNX2 and OSX were first normalized to  $\beta$ -actin levels and then calculated relative to their treatment controls.

#### Western Blot Analyses

MC3T3-E1 cells were lysed in radioimmunoprecipitation assay buffer (Solarbio, Beijing, China) supplemented with phenylmethanesulfonyl fluoride (PMSF). The total protein content was measured using a bicinchoninic acid protein assay kit (Boster, Wuhan, China). Equivalent amounts of protein from each group of cells were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck-Millipore) for 60 min at 200 mA. After blocking for 3 h with 5% nonfat dried milk in Tris-buffered saline supplemented with Tween (TBST), the PVDF membranes were incubated with antibodies against RUNX2 and OSX at ratios of 1:150 and 1:200, respectively, at 4°C overnight. Then, the membranes were incubated with secondary antibodies for 37°C for 1 h after washing the membrane

three times with TBST. The relative expression levels of the proteins of interest were normalized to that of GAPDH.

#### Outcome Measures

The icariin and H<sub>2</sub>O<sub>2</sub> dose was identified by CCK-8 assay. Oxidative stress was measured by the level of SOD, GSH, and MDA. Early and late osteogenic capacity were assessed by ALP and ARS, respectively. Osteogenic-related markers were measured by RT-PCR and western blot analyses.

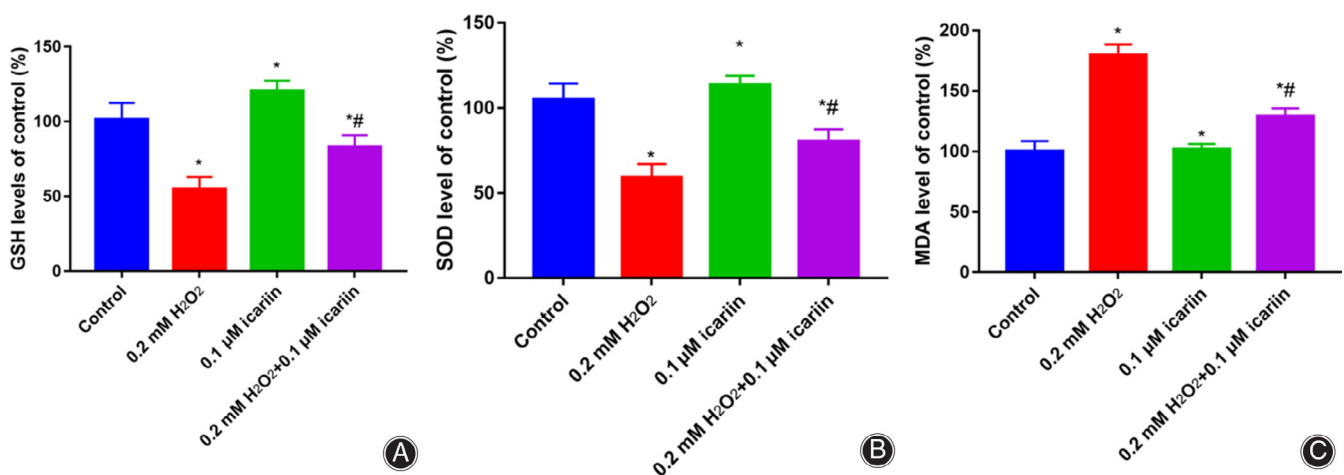
#### Statistical Analysis

The obtained data are expressed as the means  $\pm$  standard deviation. Each experiment was repeated at least three times in triplicate. Statistical analyses were performed by one-way ANOVA followed by a Bonferroni multiple comparison test for pairwise comparison. A *P*-value <0.05 was considered significant.

## Results

#### Icariin and H<sub>2</sub>O<sub>2</sub> Dose Validation

After 24 h of exposure to 0.2 mM H<sub>2</sub>O<sub>2</sub>, the viability of MC3T3-E1 cells (56.4%  $\pm$  5.6%) was significantly decreased compared to that of the control cells (100%  $\pm$  8%, *P* < 0.05, Fig. 1A). In contrast, the 0.1  $\mu$ M icariin treatment significantly increased the viability of MC3T3-E1 cells (148%  $\pm$  7%) compared to that of the control group (100%  $\pm$  8%, *P* < 0.05, Fig. 1B). We then treated MC3T3-E1 cells with both H<sub>2</sub>O<sub>2</sub> and icariin for 12, 24, 36, and 48 h to test whether icariin could alleviate H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in MC3T3-E1 cells. The results showed that the addition of icariin led to a significant recovery in the viability of MC3T3-E1 cells in a time-dependent manner (Fig. 1C).



**Fig. 2** Icariin inhibits oxidative damage of MC3T3-E1 cells. The glutathione (GSH) levels (A), superoxide dismutase (SOD) activity (B), and malondialdehyde (MDA) levels (C) in the control, 0.2 mM H<sub>2</sub>O<sub>2</sub>, 0.1  $\mu$ M icariin, and 0.1  $\mu$ M icariin combined with 0.2 mM H<sub>2</sub>O<sub>2</sub> groups. \**P* < 0.05 vs control and #*P* < 0.05 vs 0.2 mM H<sub>2</sub>O<sub>2</sub> treatment alone.

**Oxidative Stress Change**

As shown in Fig. 2, the GSH ( $55\% \pm 8\%$ ) and SOD levels ( $59\% \pm 8\%$ ) in the  $H_2O_2$ -treated group were significantly lower than those of the control group (GSH:  $101.4\% \pm 11\%$ , SOD:  $105\% \pm 9.4\%$ ). However, the MDA level in the  $H_2O_2$ -treated group ( $179.5\% \pm 9\%$ ) was significantly higher than that of control group ( $100\% \pm 9\%$ , Fig. 2).

The groups cocultured with icariin exhibited SOD and GSH levels lower than those observed in the  $H_2O_2$ -exposed group. In addition, the MDA level in the icariin and  $H_2O_2$ -treated group was significantly lower than that observed in the  $H_2O_2$ -treated group ( $P < 0.05$ ).

**Alkaline Phosphatase and Alizarin Red S Results**

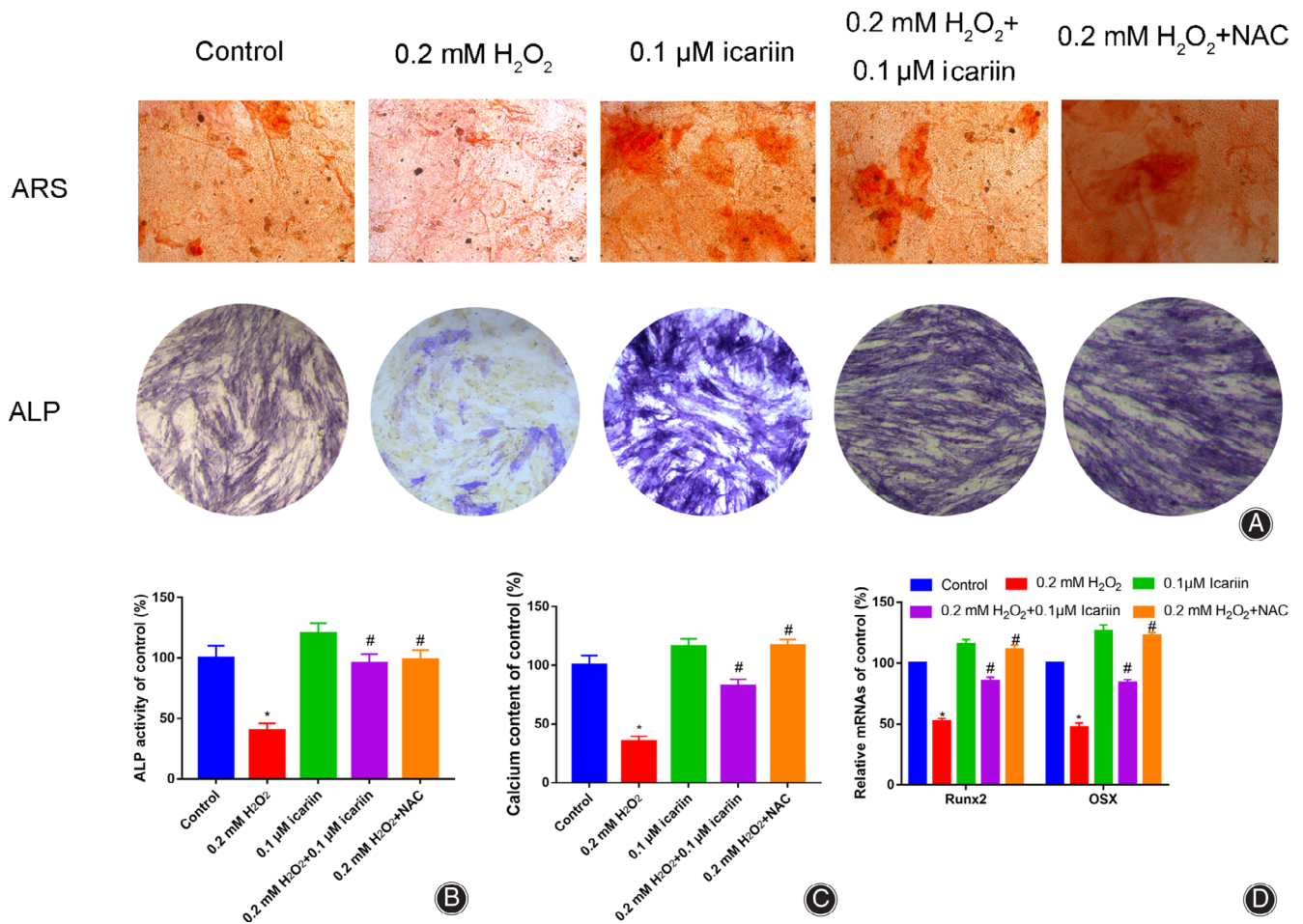
The  $H_2O_2$ -induced inhibition of osteogenesis in MC3T3-E1 cells *in vitro* was observed by microimaging of cells with

ALP and ARS staining. In addition, icariin was observed to rescue the inhibition of osteogenesis mediated by  $H_2O_2$ . Furthermore, the quantitative analysis results of the ALP activity and calcium content in each group were in accordance with the microphotography results (Fig. 3A–C).

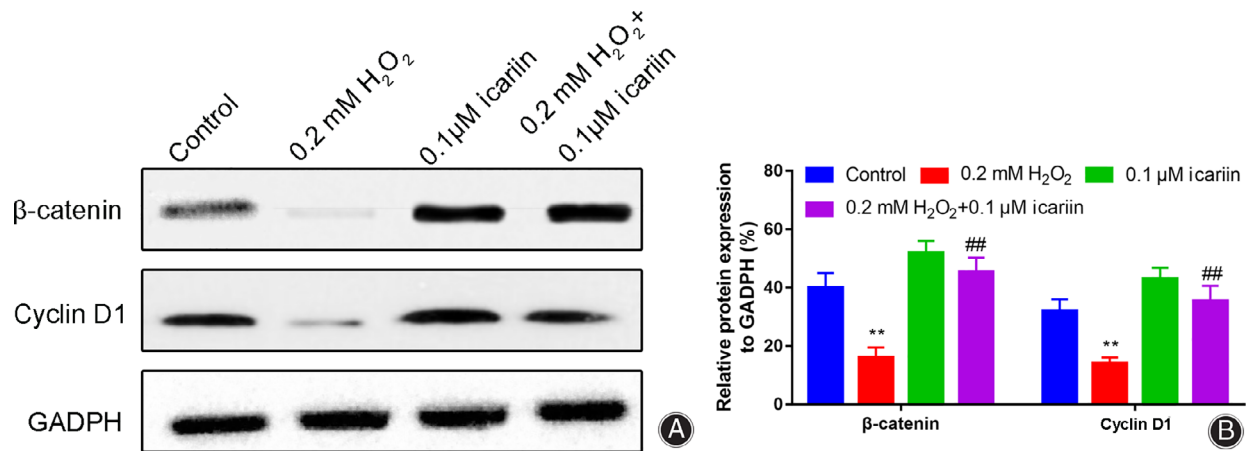
The RT-PCR results are presented in Fig. 3D and showed that compared to those in the control group, RUNX2 and OSX mRNA levels in the  $H_2O_2$ -treated group were significantly decreased. Furthermore, compared with the control group, the icariin group exhibited increased RUNX2 and OSX mRNA levels.

**Wnt-Signaling Pathway Change**

Compared with the control group, the protein levels of  $\beta$ -catenin and cyclin D1 in the  $H_2O_2$ -treated MC3T3-E1 group were significantly decreased ( $P < 0.05$ ). Furthermore,



**Fig. 3** Icariin can reverse the inhibitory effects of  $H_2O_2$  in early and late stages of MC3T3-E1 cell osteogenic differentiation, as determined by alkaline phosphatase (ALP) staining and alizarin red S (ARS), respectively. (A) Representative ALP and ARS staining images assessed by general observation in the control, 0.2 mM  $H_2O_2$ , 0.1  $\mu$ M icariin, and 0.1  $\mu$ M icariin combined with 0.2 mM  $H_2O_2$  groups and the NAC combined with 0.2 mM  $H_2O_2$  group. The relative ALP activity (B) and calcium contents (C) in each group. (D) Relative mRNA expression levels of RUNX2 and OSX in the control, 0.2 mM  $H_2O_2$ , 0.1  $\mu$ M icariin, and 0.1  $\mu$ M icariin combined with 0.2 mM  $H_2O_2$  groups and N-acetylcysteine (NAC) combined with the 0.2 mM  $H_2O_2$  group. \* $P < 0.05$  vs control and # $P < 0.05$  vs 0.2 mM  $H_2O_2$  group.



**Fig. 4** Icariin activates the WNT/ $\beta$ -catenin signaling pathway. (A). Quantitative analysis  $\beta$ -catenin and cyclin D1 protein expression levels in each group (B). \*\* $P < 0.01$  vs control, ## $P < 0.01$  vs 0.2 mM  $H_2O_2$  treatment alone.

compared with the control group, cells simultaneously treated with icariin and  $H_2O_2$  had a similar  $\beta$ -catenin and cyclin D1, with significance ( $P < 0.05$ , Fig. 4A and B).

Our results showed that the calcium content was similar in the control and the 0.2 mM  $H_2O_2$  + 0.1  $\mu$ M icariin groups. However, the calcium content was decreased in the 0.2 mM  $H_2O_2$  + 0.1  $\mu$ M icariin + DKK-1 group compared to that observed in the 0.2 mM  $H_2O_2$  + 0.1  $\mu$ M icariin group (Fig. 5A and B).

#### Osteogenic-Related Protein Expression

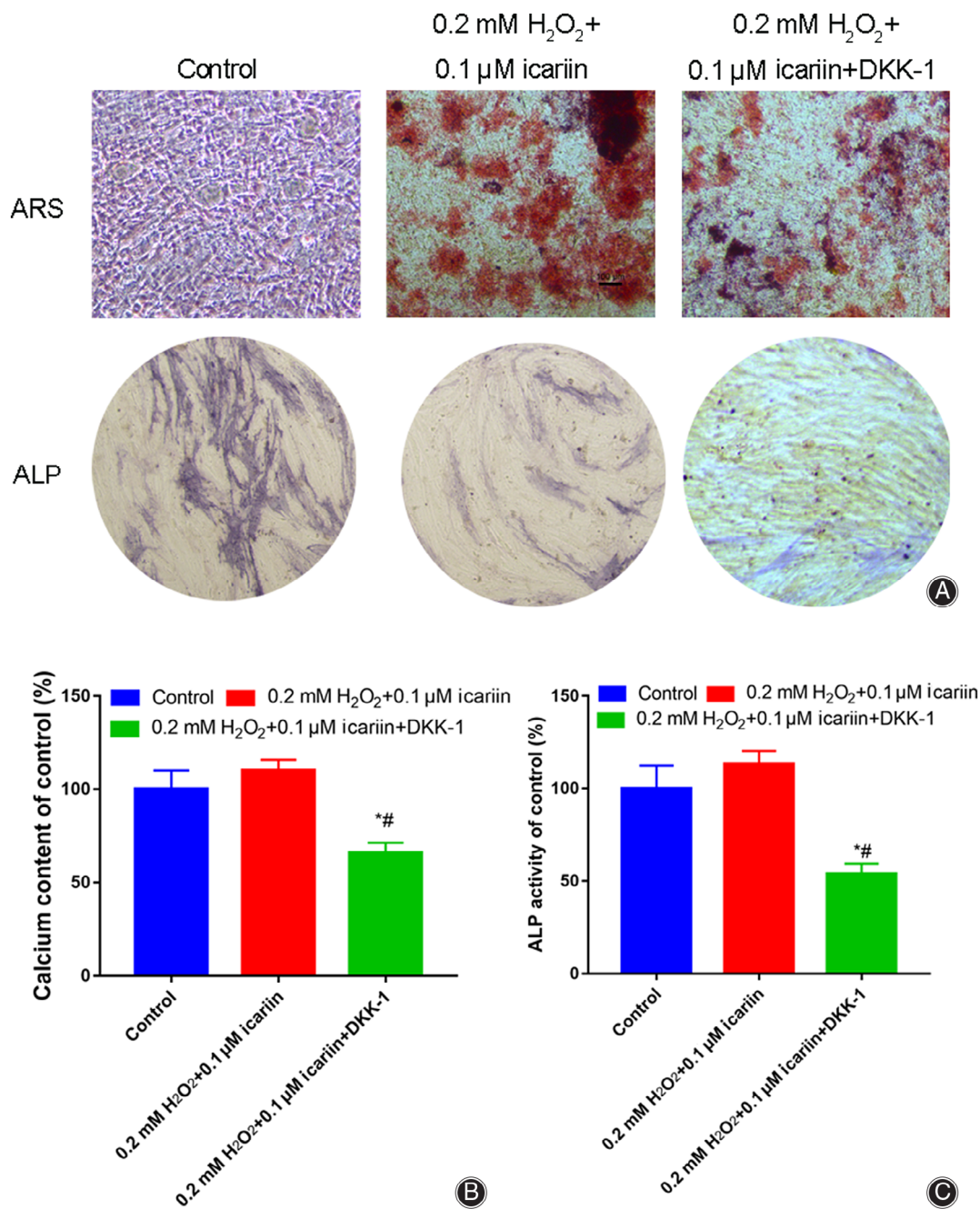
As shown in Fig. 6, the relative expression of OSX and RUNX2 was significantly decreased, by 47.1% and 59.1%, respectively, in the 0.2 mM  $H_2O_2$  + 0.1  $\mu$ M icariin + DKK-1 group than that observed in the 0.2 mM  $H_2O_2$  + 0.1  $\mu$ M icariin group ( $P < 0.05$ ). In addition, the relative expression of OSX and RUNX2 was increased by 61.7% and 68.2%, respectively, in the 0.2 mM  $H_2O_2$  + 0.1  $\mu$ M icariin group than in the control group ( $P < 0.05$ ).

#### Discussion

Our results showed that icariin has protective effects against the  $H_2O_2$ -induced inhibition of osteogenic differentiation in MC3T3-E1 cells, primarily through the Wnt-signaling pathway. First, we identified the optimal concentrations of  $H_2O_2$  and icariin using the CCK-8 assay. Then, we measured GSH, SOD, and MDA levels in these groups. We observed that icariin attenuated  $H_2O_2$ -induced oxidative stress in MC3T3-E1 cells. Furthermore, we revealed that icariin could activate Wnt signaling-relevant proteins and promote osteogenesis. In the present study, we identified a mechanism for the icariin-mediated regulation of the  $H_2O_2$ -induced inhibition of osteogenic differentiation of osteoblasts. This is the first study to show the involvement of Wnt-related genes in the oxidative stress response to icariin.

Oxidative stress greatly influences the viability, proliferation, and survival of osteocytes<sup>29</sup>. In this study, we used 0.2 mM  $H_2O_2$  to generate a model of oxidative damage according to a previous study<sup>30</sup>. We observed that after treatment with 0.2 mM  $H_2O_2$ , the viability and osteogenic differentiation capacity of cells was decreased. Furthermore, we assessed GSH, SOD, and MDA levels to confirm the oxidative stress model<sup>31</sup>. The results showed that the GSH and SOD levels in the  $H_2O_2$ -exposed group were significantly lower than those observed in the control group. In addition, the MDA levels in the icariin and  $H_2O_2$ -treated group were significantly lower than those observed in the  $H_2O_2$ -exposed group. MDA is closely associated with cell damage, and we observed that the MDA levels were significantly increased in the 0.2 mM  $H_2O_2$  treatment group compared to those observed in the control group.

We used ALP and ARS staining to assess the early and late osteogenesis capacity of cells, respectively. When cocultured with  $H_2O_2$ , ALP and ARS staining were significantly decreased. We observed that, compared with the control group, icariin could enhance ALP and ARS staining. These results indicated that icariin could rescue osteogenesis caused by oxidative damage. Wu *et al.*<sup>32</sup> controlled the release of icariin for the treatment of bone defects and observed that icariin has an antiosteoporotic effect that promotes bone defect repair. Wang *et al.*<sup>33</sup> conducted a review on the clinical practice of icariin for PO and revealed that it was effective in preventing osteoporosis with relatively few side effects. Previous studies have shown that icariin has a regulatory effect on oxidative stress in vascular endothelial cells by inhibiting endoplasmic reticulum stress<sup>34</sup>. Ding *et al.*<sup>29</sup> showed that mangiferin can inhibit oxidative damage *via* the BMP-2/Smad-1 signaling pathway. Similarly, a study conducted by Xia *et al.*<sup>35</sup> revealed that mangiferin can protect osteoblasts against oxidative damage by targeting ERK5 and Nrf2. Taken

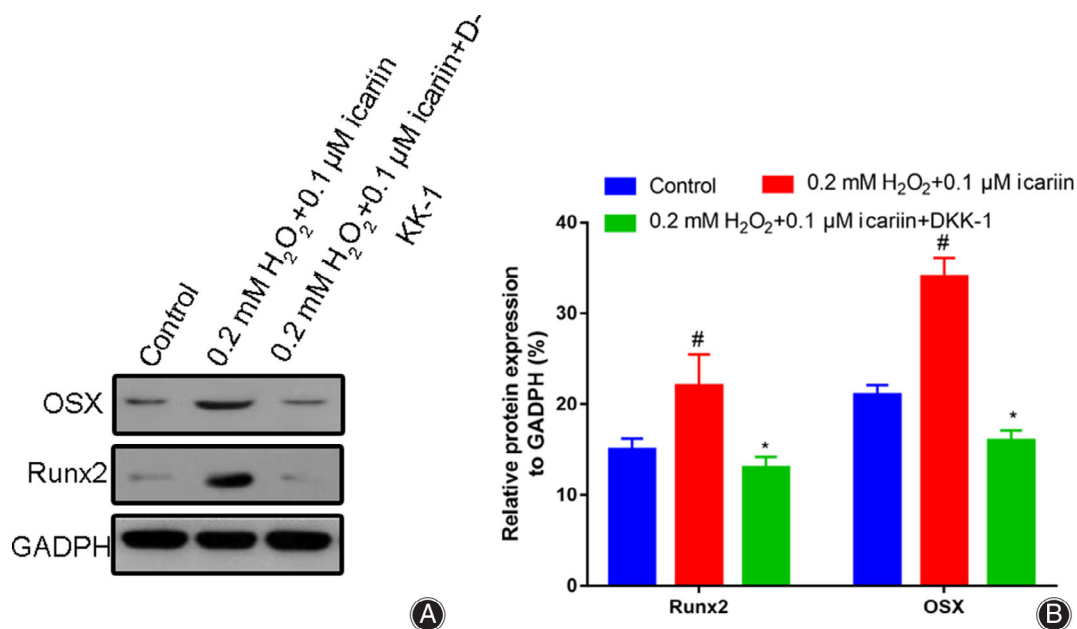


**Fig. 5** Inhibiting Wnt/ $\beta$ -catenin signaling pathway with DKK1-inhibited osteogenic differentiation of MC3T3-E1. (A). Quantitative analysis of the calcium contents (B) and alkaline phosphatase (ALP) activity (C) of each group. \* $P < 0.05$  vs control and # $P < 0.05$  vs 0.2 mM H<sub>2</sub>O<sub>2</sub> and DKK-1 treatment alone.

together, these data suggest that antioxidant components protect bone loss by reducing oxidative stress.

Wnt- $\beta$ -catenin signaling plays an important role in protection against oxidative damage induced apoptosis in MC3T3-E1 cells<sup>36</sup>. DKK-1 is an upstream antagonist of Wnt components<sup>37</sup>. DKK-1 was used to inactivate the Wnt/ $\beta$ -

catenin pathway<sup>38</sup>. We observed that when the H<sub>2</sub>O<sub>2</sub> and icariin-treated cells were treated with DKK-1, ARS staining and ALP staining decreased. In addition, we observed that in the presence of DKK-1, Runx-2, and OSX expression were decreased. Icariin can protect osteoblasts against oxidative damage by modulating Wnt signaling. Wang *et al.*<sup>24</sup> revealed



**Fig. 6** DKK-1 decreased RUNX2 and OSX protein level. A, Western blot analysis of RUNX2 and OSX expression in the control, 0.2 mM H<sub>2</sub>O<sub>2</sub>, and 0.1 μM icariin treatment groups and the group treated with of 0.2 mM H<sub>2</sub>O<sub>2</sub>, 0.1 μM icariin, and DKK-1. B, Relative protein expression of RUNX2 and OSX normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). \**P* < 0.05 vs control and #*P* < 0.05 vs 0.2 mM H<sub>2</sub>O<sub>2</sub> and DKK-1 treatment alone.

that icariin increased the mRNA and protein expression levels of β-catenin and cyclin D1, and these effects were inhibited by the Wnt/β-catenin pathway inhibitor Dickkopf-1. Liu *et al.*<sup>39</sup> showed that icariin could stimulate osteoblastic differentiation *via* the Wnt/β-catenin pathway. In addition, Zofkova *et al.*<sup>40</sup> conducted a review and suggested that icariin could stimulate Wnt/β-catenin signaling and regulate the osteoblast activity.

### Conclusion

In summary, in this study, we demonstrated that icariin increases the viability of MC3T3-E1 cells, reverses the oxidative stress induced by H<sub>2</sub>O<sub>2</sub>, and protects MC3T3-E1 cells against the H<sub>2</sub>O<sub>2</sub>-induced inhibition of osteogenic differentiation, which may occur through the Wnt signaling pathway. Future studies should focus on the specific mechanism of icariin in promoting osteogenesis in MC3T3-E1 cells.

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