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Original Article

Icaritin suppresses the proliferation of human osteosarcoma cells *in vitro* by increasing apoptosis and decreasing MMP expression

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Aim: To explore whether icaritin, a prenylflavonoid derivative of the Chinese tonic herb Epimedium, could suppress the proliferation of human osteosarcoma cells *in vitro*, and to elucidate the mechanisms of the action.
Methods: Human osteosarcoma $SaOS_2$ cell line was used in the present study. The proliferation of the cells was examined using MTT assay and immunofluorescence DAPI staining. Cell motility was studied with the scratch assay. Cell apoptosis was determined by Annexin V-FITC and PI double staining using flow cytometry. Western blotting and RT-PCR were used to measure the expression of mRNAs and proteins in the cells.
Results: Icaritin (5–15 μmol/L) suppressed the proliferation of $SaOS_2$ cells *in vitro* in a dose-dependent manner. Furthermore, the cell motility was significantly decreased after exposure to icaritin. Moreover, icaritin (5 μmol/L) time-dependently induced the apoptosis of $SaOS_2$ cells, markedly suppressed MMP-2 and MMP-9 expression, upregulated caspase-3 and caspase-9 expression, and increased the level of cleaved caspase-3 in the cells. Co-exposure to the caspase-3 inhibitor zVAD-fmk (10 μmol/L) compromised the icaritin-induced caspase-3 expression and apoptosis in $SaOS_2$ cells.
Conclusion: Icaritin suppresses the proliferation of $SaOS_2$ human osteosarcoma cells by increasing apoptosis and downregulating MMP expression.

Keywords: osteosarcoma; icaritin; apoptosis; MMP-2; MMP-9; caspase-3; zVAD-fmk

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Introduction

Icaritin, a prenylflavonoid derivative from the Chinese tonic herb *Epimedium*, has been shown to have many pharmacological and biological activities, such as antioxidant activity[1], enhancement of the osteoblastic differentiation of mesenchymal stem cells[2–4], inhibition of human prostate carcinoma growth[5, 6] and neuroprotective effects[7–9].

Osteosarcoma (OS) is one of the most common malignant bone tumors during childhood and adolescence and is the second leading cause of mortality in this age group[10, 11]. This type of bone tumor is characterized by a highly malignant and metastatic potential, and approximately 80% of osteosarcomas originate in the appendicular skeleton. Multi-drug chemotherapy has greatly increased the five year survival rate from 20% to 70%[12–14]. However, the rate has been stagnant for 30 years, and disease prognosis is particularly poor for patients with recurrent and metastatic disease, partially due to the emergence of chemotherapy resistance. The abnormal control of cell proliferation in osteosarcoma has been linked to the suppression of apoptosis[15], increased MMP expression[12, 16] and the genetic alteration of oncogenes that regulate the cell cycle[17, 18].

The mechanisms underlying the antitumor activity of icaritin have drawn increasing attention[5, 6, 18–20]. Although accumulating evidence has shown that icaritin plays a potential role in inhibiting cancer cell growth, the underlying mechanism remains elusive. It has been reported that icaritin can effectively inhibit tumor growth, such as the growth of endometrial cancer cells and human prostate carcinoma growth. Therefore, we aimed to analyze the potential antitumor mechanism of icaritin in osteosarcomas, and we provide clues for developing this compound into a novel therapy.

Materials and reagents

All reagents were of the highest purity. Icaritin was purchased from the National Institute of Control of Pharmaceuticals and

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Biological Products (Beijing, China); the product ID was 45588, and the purity was >98%, as analyzed by HPLC. A 30 μmol/L stock solution was prepared by dissolving icaritin in 10% DMSO (Sigma-Aldrich, USA) and was stored at 20 °C. The caspase inhibitor zVAD-fmk was prepared and stored at 20 °C (Selleck, USA).

Cell line and cell culture

The $SaOS_2$ human osteosarcoma cell line was obtained from the Global Bioresource Center (ATCC, Rockefeller, Maryland, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in an incubator at 37 °C in the presence of 5% CO_2.

Cell proliferation essay

Cells were seeded in a 96-well dish at a final density of 1×10^4 cells/well and were incubated in DMEM medium containing 10% FBS for 24 h. Then, the cells were treated with different concentrations (0, 1, 2, 3, 5, 10, or 15 μmol/L) of icaritin for 24, 48, and 72 h. The medium was removed, and fresh medium was added to each well along with 20 μL of MTT solution (5 mg/mL). After a 2 h incubation, 150 μL of DMSO was added to each well. The absorption at 570 nm was determined for each sample using an automatic ELISA plate reader. Three replicate wells were used for each treatment, and the experiments were repeated twice[21, 22].

Scratch assays

Cells were seeded in a 24-well dish at a final density of 1×10^5 cells/well and were incubated in DMEM medium containing 10% FBS for 24 h. The $SaOS_2$ cells were wounded with a pipette tip to obtain two perpendicular wounds that formed a cross shape. The cells in the experimental group were treated with 5 μmol/L icaritin for 0, 24, 48, and 72 h. The cells in the control group were treated with the same volume of DMSO for the same time. The wounds were photographed at 0, 24, 48, and 72 h using an inverted microscope (Leica, Frankfurt, Germany). The distance migrated was calculated by dividing the distance at the time point by the distance at the beginning. For each experiment, a total of 4 wounds were measured per group, and each experiment was repeated twice.

Immunofluorescence staining

Cells were seeded in a 24-well dish at a final density of 1×10^5 cells/well and were incubated in DMEM medium containing 10% FBS for 24 h. The cells were then treated with 5 μmol/L icaritin for 24 h; the control group was treated with the same volume of DMSO. Cells cultured on sterile glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 min and then permeabilized with 0.4% Triton X-100 for 15 min at room temperature. A polyclonal rabbit anti-MMP-2 antibody (1:250, Bioworld Technology, Nanjing, China), anti-MMP-9 antibody (1:250, Bioworld Technology, Nanjing, China), anti-caspase-3 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, USA) or anti-caspase-9 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, USA) was applied to the sections at 4 °C overnight. Next, the sections were incubated with FITC-conjugated goat anti-rabbit IgG antibody (1:200; Sigma-Aldrich, St Louis, CA, USA). Following immunostaining, all of the sections were mounted with Mowiol anti-fading agent containing DAPI to counterstain the nuclei. Images were taken using a Leica DM3000.

Western blot analysis

For Western blot analysis, the cells were seeded in a 6-well dish to a final concentration of 1×10^6 cells/well and incubated in DMEM medium containing 10% FBS for 24 h. The cells were then treated with 5 μmol/L icaritin for 24, 48, or 72 h, and the control group was treated with the same volume of DMSO. The cells were washed twice in ice cold PBS. The samples were individually homogenized in 5 mmol/L Tris–HCl buffer containing 4 mmol/L EDTA, pH 7.4, 1 μmol/L pepstatin, 100 μmol/L leupeptin, 100 μmol/L phenylmethyl sulfonylfluoride, and 10 μg/mL aprotinin, and the lysates were cleared by centrifugation at $14000\times g$ for 10 min at 4 °C. Protein concentrations were determined using the Lowry method. Approximately 100 μg of protein was run on a discontinuous SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBS containing 0.05% Tween 20 and were incubated with the following primary antibodies overnight at 4 °C: polyclonal rabbit anti-MMP-2 antibody (1:250, Bioworld Technology, Nanjing, China), anti-MMP-9 antibody (1:250, Bioworld Technology, Nanjing, China), or anti-caspase-3 antibody (1:500, Cell signaling, Boston, Massachusetts, USA). The optical density (*OD*) of the signals was quantified and is expressed as the ratio of the *OD* of the tested proteins to the *OD* of β-actin.

Reverse transcription polymerase chain reaction (RT-PCR)

For PCR analysis, the cells were seeded in a 6-well dish at a final density of 1×10^5 cells/well and were incubated in DMEM medium containing 10% FBS for 24 h. The cells were then treated with 5 μmol/L icaritin for 24 h, and the control group was treated with the same volume of DMSO. Total RNA was extracted from the cultured cells using TRIzol reagent according to the manufacturer's protocol. RNA was quantified using spectroscopy at 260 nm. Five micrograms of RNA was reversely transcribed into cDNA using a standard RT protocol. One microliter (0.4 μg) of cDNA was added to a PCR reaction premix containing 10 pmol/L of each of the corresponding primer pairs. The primers used in this analysis are listed in Table 1. PCR amplification was performed over 35 cycles of 95°C for 30 s, 60 °C for 30 s, and 72 °C for 90 s. The PCR products were separated on 1% agarose gels and were stained with ethidium bromide. The amount of each product was quantified using a gel documentation system (Bio-Rad, Nanjing, China). β-Actin expression was used as an internal reference to verify equal concentrations of cDNA in each sample.

Flow cytometry

Apoptosis was analyzed using flow cytometry. For the apop-

Table 1. Primers of MMP-2, MMP-9, and β-actin.

Gene	Primers
MMP-2	Forward: 5′-GATAACCTGGATGCCGTCGTG-3′
	Reverse: 5′-CAGCCTAGCCAGTCGGATTTG-3′
MMP-9	Forward: 5′-AATCTCACCGACAGGCAGCT-3′
	Reverse: 5′-CCAAACTGGATGACGATGTC-3′
β-Actin	Forward: 5′-CGTFGACATCCGTAAAGACC-3′
	Reverse: 5′-TAGAGCCACCAATCCACACA-3′

tosis analysis, cells were incubated with 5 μmol/L icaritin for 0, 24, 48, or 72 h, followed by Annexin V-FITC and propidium iodide (PI) double staining performed according to the manufacturer's instructions (Biosea, Beijing, China).

The effect of caspase inhibitor zVAD-fmk on icaritin-induced apoptosis

Cells were seeded in a 6-well dish at a final density of 1×10^5 cells/well and were incubated in DMEM medium containing 10% FBS for 24 h. The cells were treated with icaritin alone or in combination with the caspase-3 inhibitor 10 μmol/L zVAD-fmk for 24 h, and the lysates were used for the Western blot analysis. The control group was treated with the same volume of 10% DMSO. An MTT assay was performed to investigate the effect of zVAD-fmk on the growth of the $SaOS_2$ cells.

Statistical analysis

Comparisons of the two data sets were analyzed using *t*-tests, and data with more than two variables were analyzed using two-way repeated-measures ANOVAs with *post hoc* Tukey's analysis. All data are plotted as the mean±standard deviation.

Results

Icaritin inhibits $SaOS_2$ proliferation *in vitro*

To investigate the effect of icaritin on the inhibition of $SaOS_2$ proliferation, we performed an MTT assay and immunofluorescence DAPI staining. For the MTT analysis, the cells were incubated for various lengths of time (24, 48, or 72 h) in medium containing various concentrations (0, 1, 2, 3, 5, 10, or 15 μmol/L) of icaritin. The control cells were incubated under the same conditions. Administration of 5 μmol/L icaritin significantly decreased $SaOS_2$ proliferation after 24 h, while icaritin treatment exerted inhibitory effects on the $SaOS_2$ cells starting at a dose of 3 μmol/L at 72 h (Figure 1A). For the immunofluorescence DAPI staining, we again confirmed that 5 μmol/L icaritin significantly suppressed $SaOS_2$ proliferation. The number of DAPI(+) cells was significantly reduced after 48 and 72 h of icaritin treatment compared to the number of DAPI(+) cells at 24 h ($P<0.05$). Meanwhile, a dose of 5 μmol/L icaritin for 72 h obviously decreased the number of DAPI(+) cells compared to the 48 h treatment ($P<0.05$, Figure 1B).

Icaritin regulates osteosarcoma cell motility

Scratch assays were performed to investigate the changes in cell motility in the icaritin-treated and control groups. The results showed that icaritin treatment decreased the motility of $SaOS_2$ cells after 24, 48, and 72 h of treatment compared to the control group ($P<0.05$). The average distance that the $SaOS_2$ cells in the icaritin-treated group moved was shorter than that of the cells in the control group. The ratio of the distance moved in the control group was 39.1%, 55.6%, and 78.5% at 24, 48, and 72 h. Meanwhile, for the icaritin-treated group, the ratio was 19.8%, 39.2%, and 56.1% at 24, 48, and 72 h (Figure 2A, 2B).

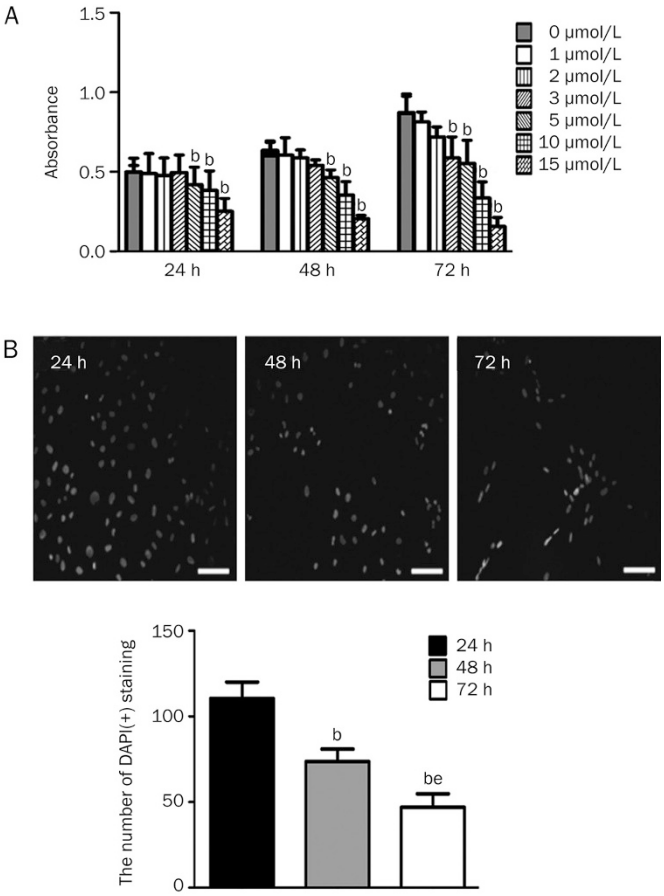

Figure 1. The effect of icaritin on $SaOS_2$ cell proliferation. The values are expressed as the absorbance (A). Nuclei staining by DAPI (B). $SaOS_2$ cells were stained with DAPI after incubation with 5 μmol/L icaritin for 24, 48, and 72 h. Scale bar=100 μm. *n*=3. Mean±SD. $^bP<0.05$ *vs* 24 h. $^eP<0.05$ *vs* 48 h.

Icaritin inhibits the expression of MMP-2 and MMP-9 in the $SaOS_2$ cell line

To assess the effect of icaritin on the expression of MMP-2 and MMP-9 in the $SaOS_2$ cells, we performed Western blot, RT-PCR and immunofluorescence staining analyses. For the immunofluorescence staining, we observed that the intensity of MMP-2 and MMP-9 expression in the $SaOS_2$ cells was very high without icaritin treatment. Then, after treatment with 5 μmol/L icaritin, we found that the levels of MMP-2 and

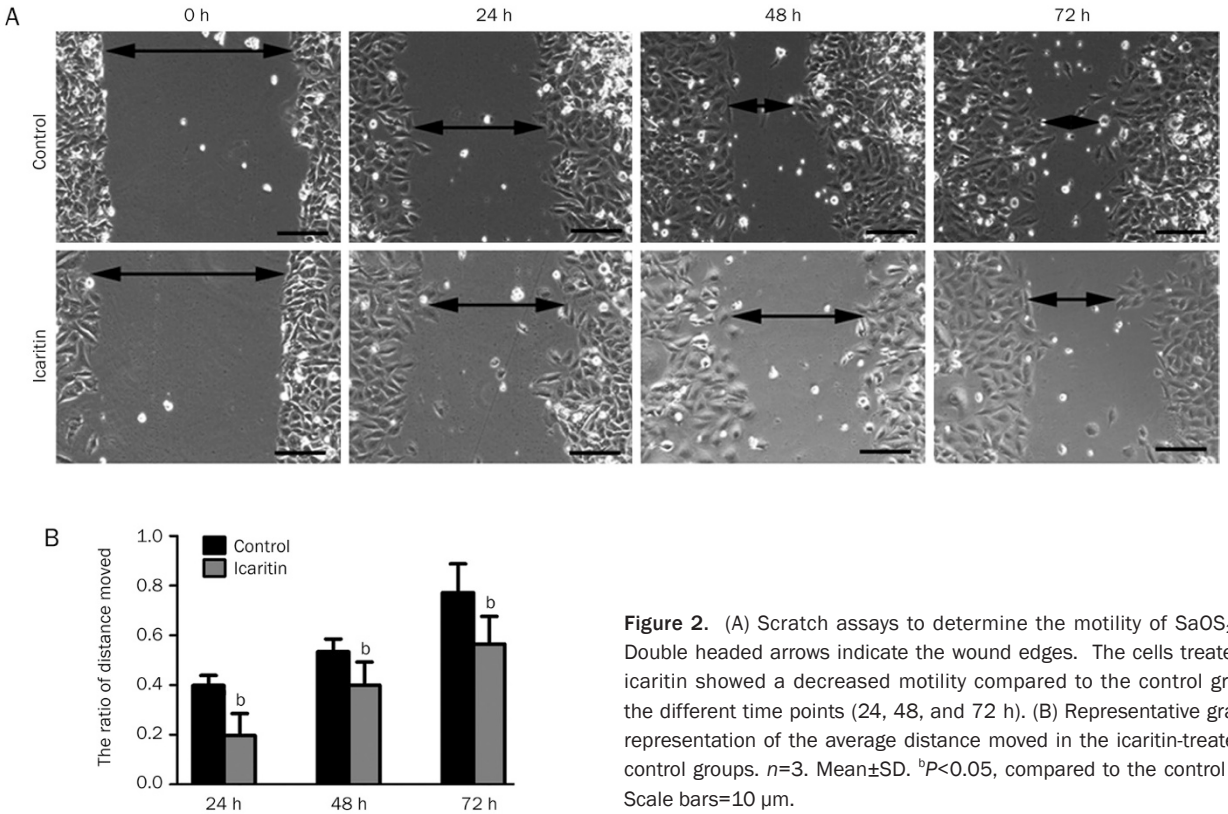

Figure 2. (A) Scratch assays to determine the motility of $SaOS_2$ cells. Double headed arrows indicate the wound edges. The cells treated with icaritin showed a decreased motility compared to the control group at the different time points (24, 48, and 72 h). (B) Representative graphical representation of the average distance moved in the icaritin-treated and control groups. n=3. Mean±SD. ^bP<0.05, compared to the control group. Scale bars=10 μm.

MMP-9 expression in the icaritin-treated $SaOS_2$ cells were significantly lower than in the control group (Figure 3). For the Western blot analysis, we observed that 5 μmol/L icaritin significantly suppressed MMP-2 and MMP-9 expression at the protein levels from 75.2% at the 24 h time point to 27.1% at the 72 h time point (P<0.05) (Figure 4). For the RT-PCR analysis, the mRNA of MMP-2 and MMP-9 was also decreased after 24 h of treatment (Figure 5).

Icaritin increases the expression of caspase-3 and caspase-9 in the $SaOS_2$ cell line

To investigate caspase-3 and caspase-9 expression after icaritin treatment, we performed immunofluorescence staining and Western blot analyses. For the immunofluorescence staining, the results showed that icaritin treatment for 24 h significantly upregulated the expression of caspase-3 and caspase-9 to increase apoptosis. For the $SaOS_2$ cells that were not treated with icaritin, the intensity of caspase-3 and caspase-9 expression was very low (Figure 6A). For the Western blot analysis of cleaved caspase-3, we found that icaritin treatment increased the levels of cleaved caspase-3 by approximately 4-fold after 24, 48, and 72 h compared to the control group (Figure 6B and 6C).

The flow cytometry analysis for apoptosis induced by 5 μmol/L icaritin treatment

To reveal the effect of icaritin on the apoptosis rate of $SaOS_2$ cells, we performed flow cytometry following AnnexinV-FITC and PI double staining. The percentage of apoptotic cells after icaritin treatment for 24, 48, and 72 h (9.9%, 16.5%, and 23.8%) was obviously increased compared with to that of the control group (Figure 7A and 7B).

The effect of the caspase-3 inhibitor zVAD-fmk on the icaritin-induced apoptosis

To investigate the effect of the caspase inhibitor zVAD-fmk on the icaritin-induced apoptosis, we performed Western blot analysis to evaluate the levels of cleaved caspase-3. We found that the caspase inhibitor zVAD-fmk could decrease the icaritin-induced caspase-3 expression in the zVAD-fmk+icaritin-treated group compared to the icaritin-treated group (P<0.05) (Figure 8A, 8B). For the MTT assay, we found that zVAD-fmk could compromise the icaritin-induced apoptosis at 24, 48, and 72 h. The absorbance of the zVAD-fmk+icaritin-treated group at 24, 48 and 72 h was higher compared to that of the icaritin-treated group at 24, 48, and 72 h (P<0.05, Figure 8C).

Discussion

The current study was the first to systemically demonstrate that icaritin, which is extracted from the Chinese tonic herb *Epimedium*, enhanced the caspase-dependent apoptosis and suppressed the motility and MMP-2/9 expression in the $SaOS_2$ human osteosarcoma cell line *in vitro*. Therefore, icaritin may have good therapy prospects for treatment of osteosarcoma patients.

Icaritin has been shown to inhibit growth and induce apop-

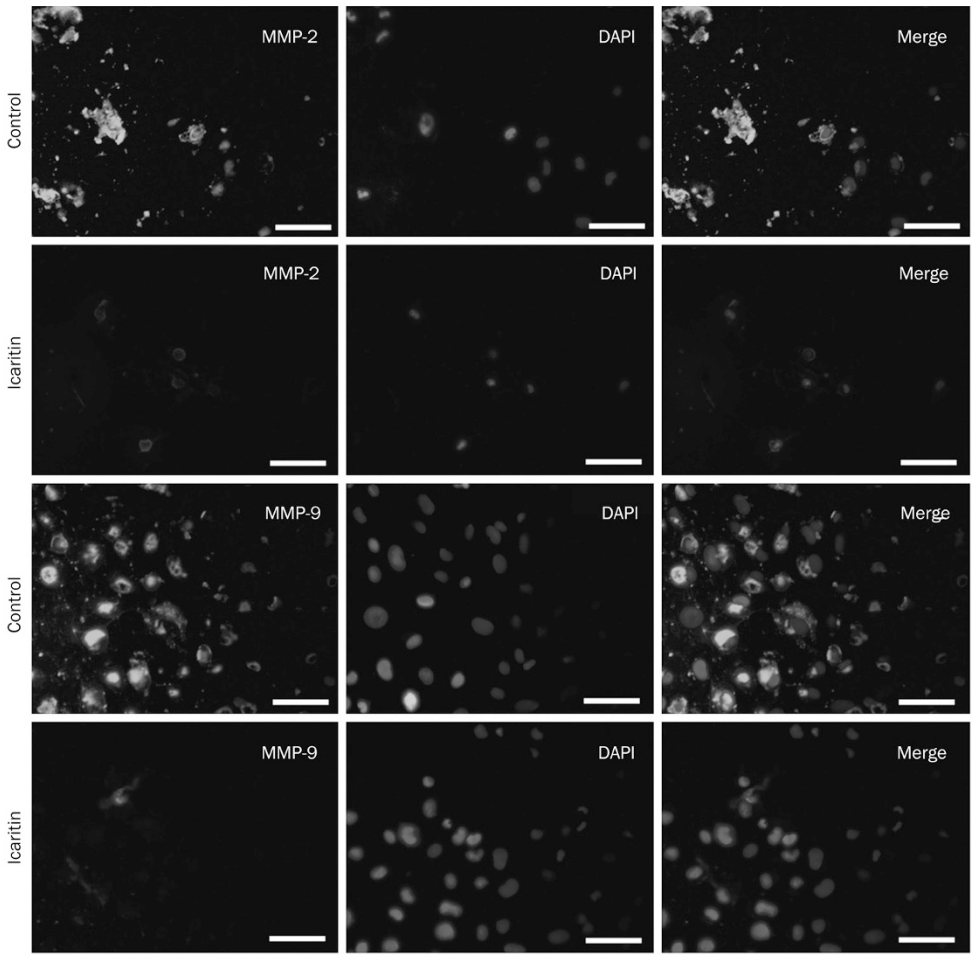

Figure 3. Immunofluorescence staining analysis for MMP-2 and MMP-9 expression in the $SaOS_2$ cells after 24 h of treatment. The immunofluorescence staining showed that the expression levels of MMP-2 and MMP-9 were significantly decreased after treatment with 5 μmol/L icaritin compared to the control group. Scale bar=50 μm.

tosis in many tumor cells, such as human endometrial cancer cells[6], $HepG_2$ cells[20], and human prostatic smooth muscle cells[19]. Icaritin caused sustained ERK1/2 activation and induced apoptosis in human endometrial cancer cells, and icaritin could also induce apoptosis in HepG2 cells via the JNK1 signaling pathway. Icaritin may be useful as an alternative therapeutic choice for imatinib-resistant forms of CML[23]. The literature has shown that icaritin can suppress the proliferation of many types of cells. The antitumor effect was not specific to human osteosarcoma cells. In our study, we revealed that icaritin could suppress cell growth and induce apoptosis in the $SaOS_2$ human osteosarcoma cell line (Figure 1 and 7).

Metastasis involves many important processes that enable tumor cells to invade into the surrounding tissues, including the coordination of several signaling pathways that allow the detachment of tumor cells, the degradation of the extracellular matrix, invasion, migration, adhesion to endothelial cells, and the reestablishment of tumor growth at a distant site[24]. It is now well established that metalloproteases have a major influence on the development, invasion, and metastasis of cancer. The metalloproteases are divided into six categories: collagenase (MMP-1, MMP-8, and MMP-13), gelatinase (MMP-2, MMP-9), interstitial lysing (MMP-3, MMP-7, and MMP-10), membrane type matrix metalloproteinase (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), lytic enzyme substrate (MMP-7, MMP-26) and other types[25–28]. Because MMP-2 and MMP-9 are the major matrix metalloproteinases (MMPs), their activity is increased in many malignant cancers. MMP-2 and MMP-9 also play key roles in tumor cell invasion and metastasis due to their ability to degrade type IV collagen, a major component of the ECM[29–31].

Overexpression of various MMPs, particularly MMP-2, is correlated with poor prognosis in many types of cancer, including adrenocortical cancer, breast cancer, and thyroid malignancies[32, 33]. In many types of tumors, the expression level of MMP-9 is markedly higher than in normal tissues[34, 35]. Moreover, patients with higher MMP-9 expression have a significantly shorter overall survival time than patients with low MMP-9 expression[36]. In our study, the observed inhibitory effects of icaritin on the expression of MMP-2 and MMP-9 in $SaOS_2$ cells suggested that icaritin may modulate the inva-

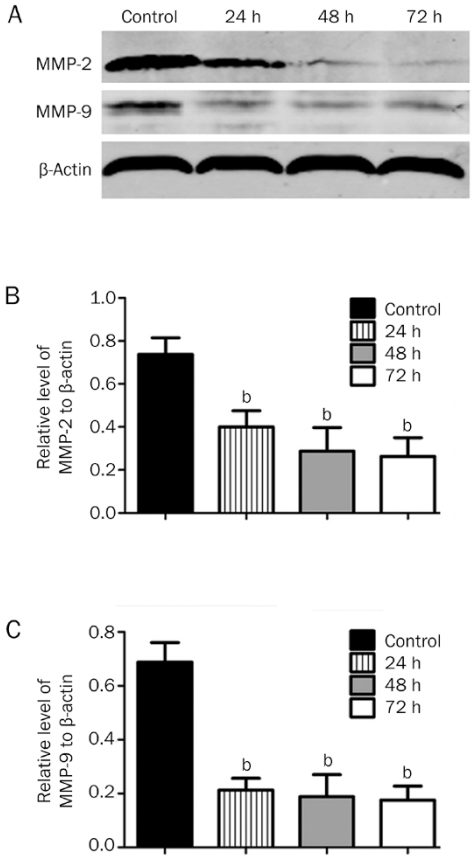

Figure 4. Western blot analysis of MMP-2 and MMP-9 expression in the $SaOS_2$ cells after 24, 48, and 72 h of treatment. Western blot analysis confirmed that icaritin could significantly downregulate MMP-2 and MMP-9 expression at 24, 48, and 72 h. n=3. Mean±SD. $^bP<0.05$ *vs* the control group. β-Actin protein levels indicate that an equal amount of protein was loaded into each lane.

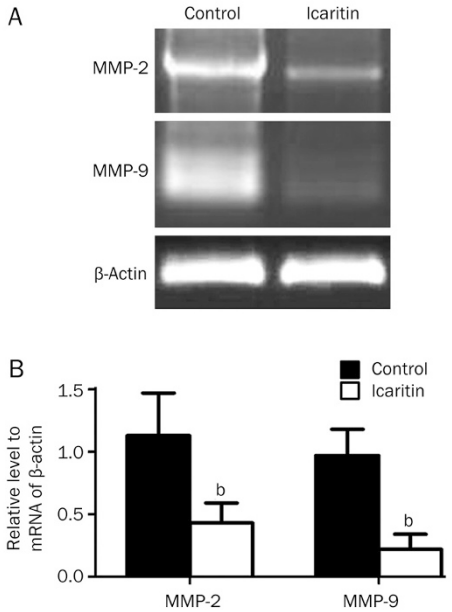

Figure 5. RT-PCR analysis of MMP-2 and MMP-9 mRNA expression in the $SaOS_2$ cells after 24 h of treatment. RT-PCR analysis confirmed that icaritin could significantly decrease the mRNA expression of MMP-2 and MMP-9. n=3. Mean±SD. $^bP<0.05$ *vs* control group.

sion and migration of osteosarcoma cells (Figure 3–5). In the scratch assays used to determine the motility of the $SaOS_2$ cells, the cells treated with icaritin had decreased motility compared to those in the control group (Figure 2). Osteosarcoma patients frequently undergo lung and liver metastases. Based on the inhibitory effects of icaritin on the expression of MMP-2 and MMP-9, icaritin may have potential as a treatment for patients with advanced osteosarcoma.

According to previous studies, the mechanisms underlying the antitumor activity of icaritin include the induction of apoptosis by multiple signaling pathways[5, 6, 20, 23]. The caspase (cysteinyl aspartate specific protease) family is involved in apoptotic signal transduction and plays an important role in regulating apoptosis[37, 38]. Caspase-3 is the final performer, and zVAD-fmk inhibits its activity and blocks cell death. The mechanism by which zVAD-fmk blocks caspase activity is through the induction of caspase-3 alkylation and the suppression of its ability to induce apoptosis[39]. In our study, we tested whether the icaritin-induced apoptosis was caspase-dependent using the caspase inhibitor zVAD-fmk. The results revealed that the effect of icaritin-induced apoptosis was decreased when the cells were treated with icaritin and zVAD-fmk. This finding illustrated that zVAD-fmk could override the icaritin-induced apoptosis and increase $SaOS_2$ growth (Figure 8C). Thus, these results illustrated that the icaritin-induced apoptosis was associated with the caspase cascade. Caspase-3 and caspase-9, which are the key factors in the caspase cascade, were upregulated after icaritin treatment for 24 h. These findings demonstrated that icaritin treatment increased both the apoptosis rate of the tumors and the activation of caspase-3 and caspase-9.

It has been reported that icaritin is relatively stable in the stomach. However, the drug begins to be metabolized in the intestine[40]. The oral bioavailability of icaritin in rats was found to be low, and first-pass metabolism might exist. A large amount of icariside II, which showed more potential pharmacological activity than icaritin, was found in rat plasma after intragastric administration of icaritin[41]. One study reported that icaritin has many metabolized products, including icariside I, icariside II, and icariside II 7-o-GLC UA. Icariside II, which is the degradation product of icaritin, is absorbed in the intestine and then undergoes C_7-OH glucuronidation in the liver. The glucuronic acid product is released into the small intestine via the bile. Meanwhile, icariside II, which is not metabolized, is released into the urine through the kidneys. Thus, a literature review revealed that the first-pass effect of icaritin exists in the body. In our study, we revealed that the antitumor effect of icaritin is obvious. However, taking the first-pass effect of icaritin into consideration, a special modified method should be used to reduce the extent of the hepatic first-pass effect.

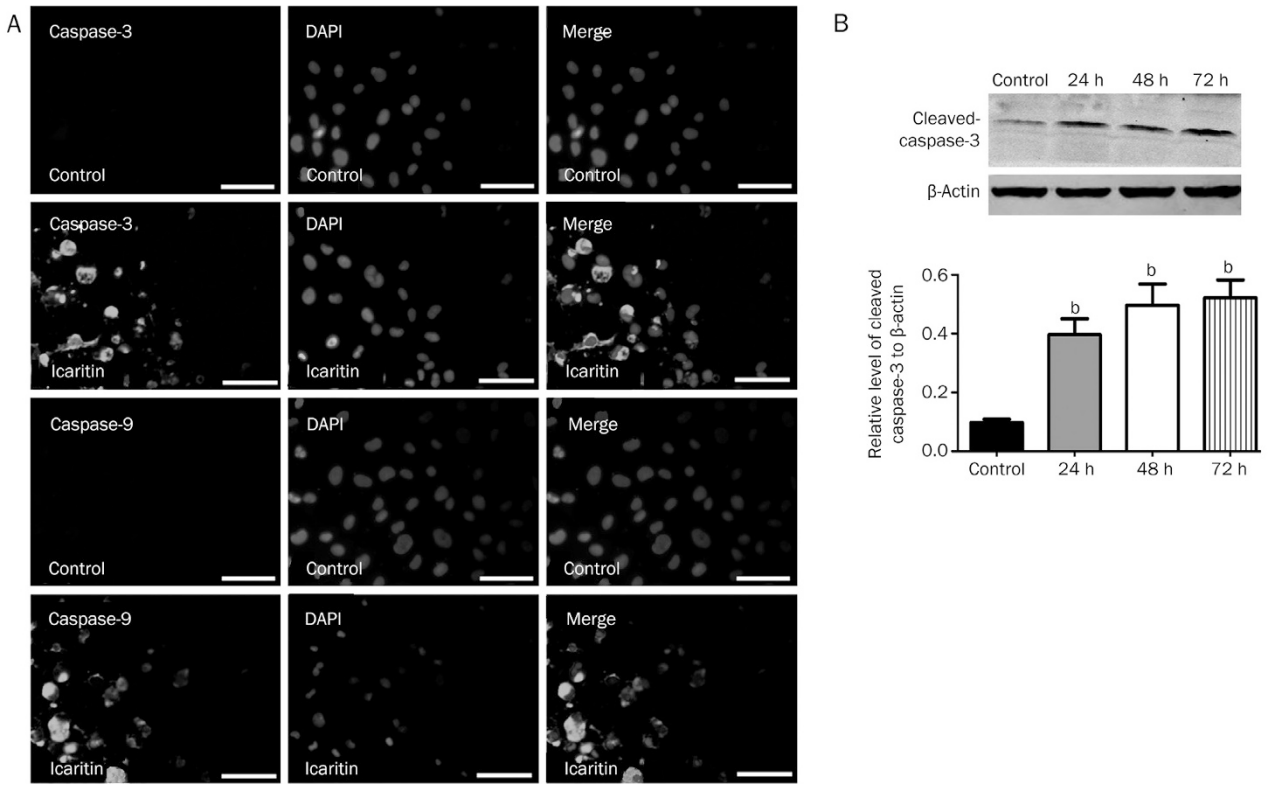

Figure 6. (A) Immunofluorescence staining analysis for caspase-3 and caspase-9 expression after 24 h of treatment. Immunofluorescence staining analysis revealed that treatment with 5 mol/L icaritin could increase the expression of caspase-3 and caspase-9 in the $SaOS_2$ cells. Scale bar=50 μm. (B and C) Western blot analysis of cleaved caspase-3. The expression of cleaved caspase-3 was significantly upregulated after 24, 48, and 72 h compared to the control group. $^bP<0.05$, compared to the control group. n=3. Mean±SD. β-Actin protein levels indicate that an equal amount of protein was loaded into each lane.

There are some limitations to our findings. In our study, we did not investigate the *in vivo* effect of icaritin on osteosarcomas. Thus, in future research, we will assess the antitumor effect of icaritin in an animal model. Furthermore, we could not reveal the mechanism by which icaritin suppresses MMP-2 and MMP-9 expression in the osteosarcoma cells. In further studies, we will investigate the underlying mechanisms of how icaritin affects MMP-2 and MMP-9 expression and the antitumor effect of icaritin *in vivo*.

In conclusion, we found that icaritin inhibited cell growth and could induce apoptosis and suppress MMP-2 and MMP-9 expression in the $SaOS_2$ human osteosarcoma cell line *in vitro*. We speculated that the underlying mechanisms involved in the icaritin-induced apoptosis were dependent on the initiation of a caspase cascade. Moreover, icaritin suppressed the proliferation of $SaOS_2$ cells by increasing apoptosis and decreasing cell motility through the downregulation of MMP-2 and MMP-9 expression.

Acknowledgments

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Author contribution

Jun WANG designed research; Xiao-fang WANG performed research, analyzed data and wrote the paper.

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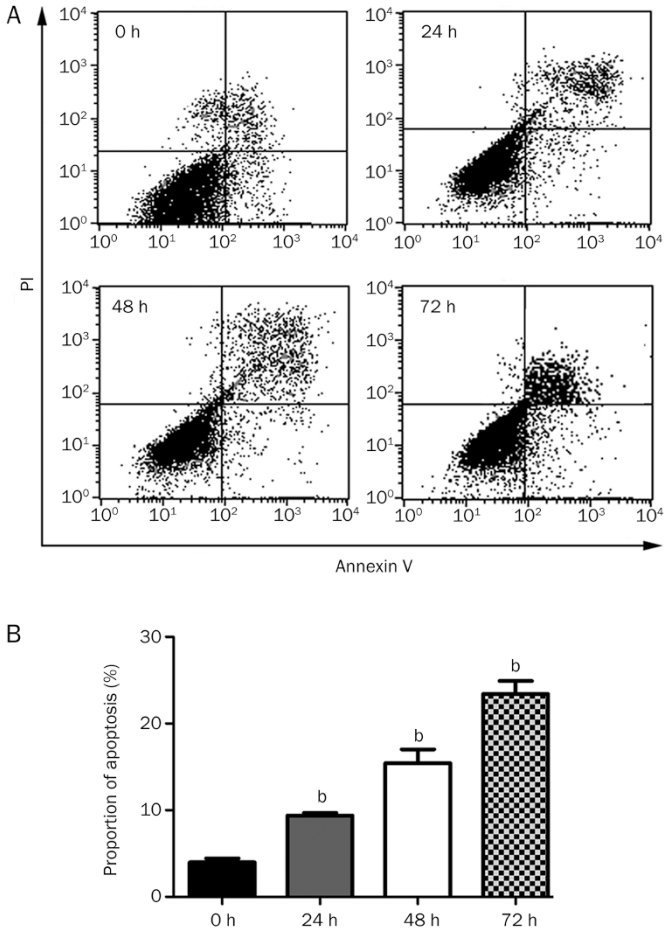

Figure 7. Flow cytometry analysis of the apoptosis rate of $SaOS_2$ cells after icaritin treatment. Apoptosis-inducing effect of CEP on $SaOS_2$ cells. After treatment with 5 μmol/L icaritin for 0, 24, 48, and 72 h, the apoptotic rates of $SaOS_2$ cells were determined by Annexin V-FITC and PI double staining using flow cytometry. n=3. Mean±SD. ^bP<0.05, compared to the control group.

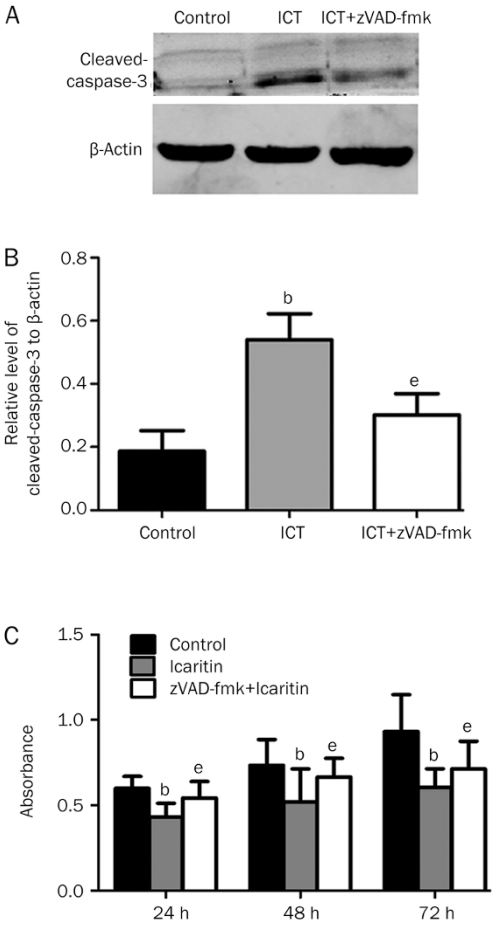

Figure 8. (A and B) Western blot analysis examining the effect of zVAD-fmk on the icaritin-induced apoptosis. The caspase inhibitor zVAD-fmk could significantly decrease the icaritin-induced cleaved caspase-3 expression. β-Actin protein levels indicate that an equal amount of protein was loaded into each lane. (C) An MTT assay examining the effect of zVAD-fmk. n=3. Mean±SD. ^bP<0.05, compared to the control group. ^eP<0.05, compared to the icaritin-treated group.

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