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Anticancer Properties of Icariside II in Human Oral Squamous Cell Carcinoma Cells

In-Ryoung Kim, Young-Seok Kim, Su-Bin Yu, Hae-Mi Kang, Hyun-Ho Kwak and Bong-Soo Park*

Department of Oral Anatomy, School of Dentistry, Pusan National University

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OSCC is currently the most common malignancy of the head and neck, affecting tens of thousands of patients per year worldwide. Natural flavonoids from plants are potential sources for novel anti-cancer drugs. Icariin is the active ingredient of flavonol glycoside, which is derived from the medical plant Herba Epimedii. A metabolite of icariin, icariside II exhibits a variety of pharmacological actions, including anti-rheumatic, anti-depressant, cardiovascular protective, and immunomodulatory functions. However, the exact mechanism causing the apoptosis-inducing effect of icariside II in OSCC is still not fully understood. In the present study, we assessed the anti-cancer effect of icariside II in OSCC cell lines by measuring its effect on cell viability, cell proliferation, and mitochondria membrane potential (MMP). Icariside II treatment of OSCC cells resulted in a dose- and time-dependent decrease in cell viability. Hoechst staining indicated apoptosis in icariside II-treated HSC cells. Icariside II inhibited cell proliferation and induced apoptosis in HSC cells, with significant increases in all present parameters in HSC-4 cells. The results clearly suggested that icariside II induced apoptosis via activation of intrinsic pathways and caspase cascades in HSC-4 cell lines. The collective findings of the study suggested that Icariside II is a potential treatment for OSCC; in addition, the data could provide a basis for the development of a novel anti-cancer strategy.

Key words: Flavonoid, Icariside II, Apoptosis, Intrinsic pathway, OSCC

Introduction

Oral cancer and, most commonly, oral squamous cell carcinoma (OSCC) is currently the most common human malignancy of the head and neck, affecting over 500,000 patients per year worldwide [1, 2]. OSCC accounts for approximately 3% of all cases of cancer. Until recently, the five-year survival rate for OSCC was below 50%. Common cancer treatments, including surgery, radiation therapy, and chemotherapy, are often used to treat OSCC. However, there are still high death rates associated with these therapies [3-5]. Therefore, new therapeutic approaches using natural agents for the treatment of OSCC are currently being investigated. The use of natural agents seems to be one of the most promising anti-cancer treatments currently in development.

Apoptosis is the physiological process of controlled elimination of unhealthy or damaged cells [6]. When a cell undergoes apoptosis, it shows nuclear condensation, DNA fragmentation, and membrane blebbing through various

*Correspondence to: Bong-Soo Park, Department of Oral Anatomy, School of Dentistry, Pusan National University, Yangsan, 626-870, Korea.
Tel.: +82-51-510-8242, Fax: +82-51-510-8241
E-mail: parkbs@pusan.ac.kr
ORCID: 0000-0002-9799-5627

pathway signals [7, 8]. The mitochondria signaling pathway is one of the apoptosis pathways. Mitochondria play an important role in the regulation of cell apoptosis. Changes in the mitochondria membrane potential (MMP) are considered an early apoptosis event, and many pro-apoptotic proteins can be released from the mitochondria into the cytoplasm when the MMP is damaged [9]. Recent studies have reported that apoptosis might play an important role in various diseases. Cancer, in particular, seems to be closely related to apoptosis. Thus, in order to develop anti-cancer drugs with less side effects than are associated with the current cancer treatment options, it is important to study the anti-cancer capacities of natural compounds [10].

Natural products and herbal medicines derived from plants may be potential sources for new anti-cancer drugs [11]. Flavonoids are polyphenols found in many foods, such as vegetables, fruit, red wine, and tea. These polyphenols are well known for their antioxidant, anti-inflammatory, anticancer, and antibacterial properties [12, 13]. Icariin is the active ingredient of the flavonol glycoside derived from the medical plant Herba Epimedii [14]. Icaritin and icariside II are metabolites of icariin [15, 16]. These products have been previously demonstrated to exhibit a variety of pharmacological activities, including anti-rheumatic, antidepressant, cardiovascular protection and immunomodulatory activities [16, 17]. The anti-tumor properties of icariin and its glycosides have recently attracted a great deal of attention from medical researchers [18-20]. However, the exact mechanism of the apoptosis-inducing effect of icariin and its glycosides in human OSCC is still not fully understood. In the present study, we demonstrate that icariin, icaritin, and icariside II are potent agents against OSCC, and that their anti-cancer functions derive from the signaling of apoptosis through the mitochondrial pathway.

Materials and Methods

Reagents

The following reagents were obtained commercially: 3-[4,5-dimethylthiazol-2-yl]2, 5-diphenyl tetrazolium bromide (MTT), and acridine orange were purchased from Sigma (St. Louis, MO, USA). Antibodies against the caspase-3 cleaved form of caspase-3 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). The AIF, Bcl-2, BAK, cytochrome c, caspase-9, ICAD, GAPDH mouse anti-rabbit IgG antibody, and rabbit anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were purchased from Sigma, unless otherwise specified.

Cell Culture

HSC-2, -3, -4 cells were kindly supplied by Prof. Sung-Dae Cho of Chonbuk National University, Jeonju, Korea. The cells were cultured in Minimum Essential Medium/Earle's Balanced Salt Solution (MEM/EBSS) supplemented with 10% FBS and 1% penicillin streptomycin (GIBCO-BRL, Rockville, MD, USA) under CO_2 humidified at 5%.

Treatment of Icariin, Icaritin, and Icariside II

The original medium was removed and icariin, icaritin, and icariside II (100 mM) stock solution were added to the medium to obtain 10 to 100 μM of these three supplements. Icariin, icaritin, and icariside II (100 mM each) were prepared in DMSO and stored in a frozen state at -20°C. When needed, the icariin, icaritin, and icariside II stock solutions were diluted to the indicated concentration with MEM/EBSS.

Cell Viability Assay

The cells were seeded on a 96-well microtiter plate (1×10^4 cells/well) and then treated with different concentrations of icariin, icaritin, and icariside II (10 to 100 μM). The cells were then incubated for different time periods. Next, the existing medium was removed and 100 μl of MTT solution (500 μg/mL) was added to each well. The cells were incubated for 4 h at 37°C. The reaction was stopped by the addition of DMSO (150 μl/well) and constantly shook for 10 min. The cell viability was monitored on an ELISA reader (Tecan, Mänedorf, Switzerland) at an excitatory emission wavelength of 620 nm.

Hoechst Staining for Morphological Aspects of Apoptosis

The number of cells undergoing apoptosis following treatment with icariside II was quantified by Hoechst staining. Different concentrations of icariside II (10, 25, and 50 μM) were treated in HSC-2, -3, and -4 cells for 24 h. After treatment, the HSC-2, -3, and -4 cells were harvested and cytocentrifuged onto clean, fat-free glass slides using a cytocentrifuge. The cells were stained with Hoechst 33342 (1

μg/ml) for 10 min at 37°C. After being washed with phosphate-buffered saline (PBS) and mounted with glycerol, the cells were observed using an inverted fluorescence microscope (Carl Zeiss, Goettingen, Germany). The untreated cell nuclei showed dispersion and uniform fluorescence, while the apoptotic cells were characterized by nuclear shrinkage, condensation, and fragmentation.

Measuring DNA Contents and MMP Using FACS

The DNA contents of HSC-2, -3, and -4 were analyzed using FACS. The cells were seeded in 100 mm culture dishes (2×10^6cells/dishes) and incubated for 24 h. After the icariside II treatments, the cells were harvested and centrifuged at 3000 rpm for 5 min. Next, they were fixed overnight in 95% ice-cold ethanol with 0.5% Tween 20, then washed in 1% bovine serum albumin PBS solution and re-suspended in PBS containing 50 μg/mL RNase A. Finally, they were incubated at 4°C for 30 min. The cells were stained with propidium iodide (50 μg/ml), and the stained cells were measured using a CYTOMICS FC500 flow cytometer system. The data was analyzed using Multi Cycle software, which allowed a simultaneous estimation of cell-cycle parameters and apoptosis.

In order to measure the MMP, the HSC-4 cells were seeded onto 60 mm dishes and incubated for one day. After being treated with icariside II for 24 h, the collected cells and $DIOC_6$ were stained directly onto the cell culture medium (final concentration=1 μM) and incubated for 30 min. The MMP was analyzed with a flowcytometry system (Beckman Coulter, CA, USA).

Western Blot Analysis

The cells were harvested and washed in ice-cold PBS, then re-suspended in 200 μl of ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris-Cl [pH 7.6], 0.5% Triton X-100, 2 mM PMSF, 2 μl/ml aprotinin, and 2 μl/ml leupeptin). The lysates were centrifuged at 13,200 rpm for 30 min at 4°C. The protein concentrations of the cell lysates were determined using a Bradford protein assay (Bio-Rad, Richmond, CA, USA). The lysates resolved on sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred, and the membrane was blocked with 5% skim milk for 2 h at room temperature. After the blocking was complete, the membranes were incubated overnight at 4°C with their respective primary antibodies. The membranes were washed 6 times for 1 h, and they reactivated secondary antibodies for 2 h at room temperature. After six washes, the membranes were detected using a Super Signal West Femto (Pierce, Rockford, IL, USA) enhanced chemiluminescence substrate before being detected with an Alpha Imager HP (AlphaInnotech, SantaClara, USA).

Fluorescence Microscopy

The cells were grown on coverslips and treated with icariside II. After 24 h, the cells were incubated with a Mitotracker at 37°C for 30 min before being fixed with 4% paraformaldehyde at room temperature for 10 min. The cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The coverslips were blocked with 3% BSA (in PBS) and incubated with the primary antibody (1:200) at room temperature for 2 h. The coverslips were then washed extensively with PBS and incubated with a FITC-conjugated secondary (1:200) antibody for 2 h at room temperature. They were then washed again in PBS and mounted with 90% glycerol. Finally, the coverslips were sealed. The cells were then visualized by laser scanning confocal microscopy. The cellular fluorescence changes were obtained using a confocal microscope LSM 700 (Carl Zeiss, Germany).

Statistical Analysis

The statistical analysis data were expressed as ± SD from at least three independent experiments. A one-way ANOVA test was used to analyze the data regarding cell viability, DNA contents, and nuclear condensation ratio on a GraphPad Prism 5.0 (GraphPad Prism Software, San Diego, CA, USA). P values less than 0.05 were considered to be statistically significant.

Results

Cytotoxic Effects of Icariin, Icaritin, and Icariside II on OSCC Cell Lines

The MTT assay was used to measure the effect of icariin, icaritin, and icariside II on the viability of OSCC cell lines (HSC-2, HSC-3, and HSC-4). First, the effects of icariin, icaritin, and icariside II on the OSCC cell lines were investigated. Next, various concentrations of icariin, icaritin, and icariside II (10 to 100 μM) were cultured for 24 h. As shown in Fig. 1, icaritin, and icariside II reduced the cell

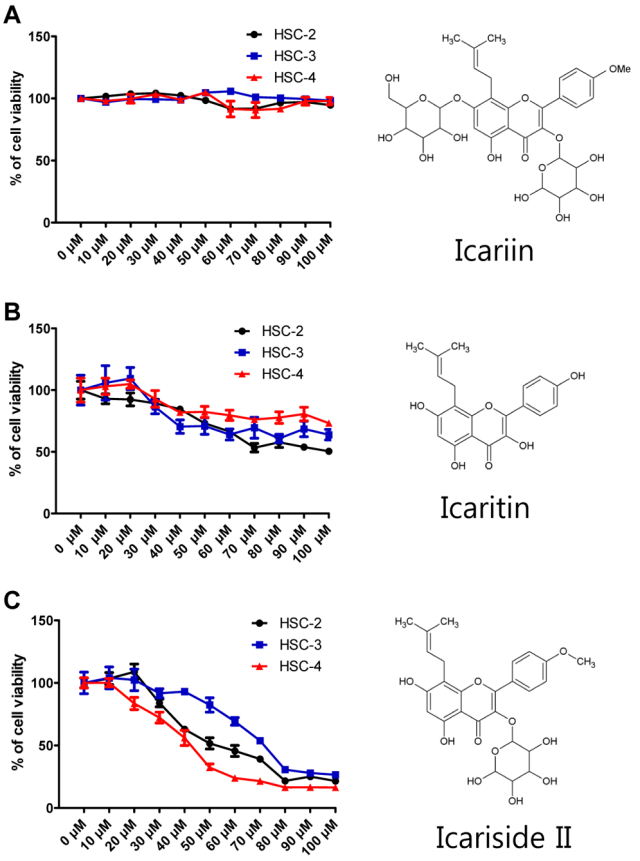

Fig. 1. Effects of icariin, icaritin, and icariside II treatment on the viability of OSCC cells. (A) Icariin, (B) icaritin, and (C) icariside II (10–100 μM) were used as treatments in three types of OSCC cells (HSC-2, -3 and 4) for 24 h. The icariside II treatment group (C) showed dramatically decreased cell viability. The cell viability was analyzed using the MTT assay. Each value represents the mean of three independent experiments ± SD (n=6).

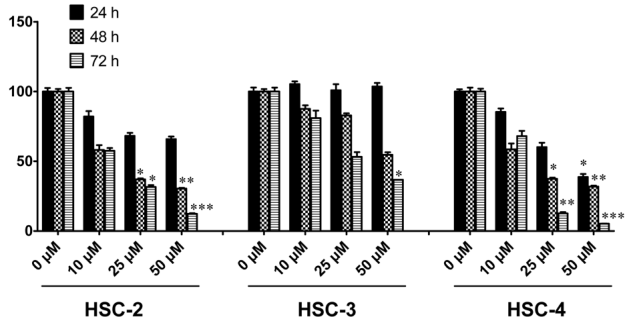

Fig. 2. The icariside II treatment clearly inhibited the cell viability of HSC-4. Icariside II (10, 25, and 50 μM) was used as treatment in three types of OSCC cells (HSC-2, -3, -4) for 24 to 72 h. Icariside II clearly inhibited the HSC-4 cell viability from 24 h to 72 h. The cell viability was analyzed using the MTT assay. Each value represents the mean of three independent experiments ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001 when compared with the control group.

viabilities of all OSCC cells excepting icariin. Icariside II, in particular, significantly decreased the cell viability depending on the dose and time (Fig. 2). For this reason, icariside II was used to conduct the next experiment.

Icariside II Increased the Apoptosis of OSCC Cells

To determine whether the cytotoxicity of icariside II occurred by apoptosis, morphological changes were conducted and the DNA contents of the nucleus were withdrawn using hoechst staining and FACS. The percentage of apoptosis occurring in the OSCC (HSC-2,-3,-4) cells was measured following treatment of the cells with increasing concentrations (0–50 μM) of icariside II. Treatment with icariside II resulted in an

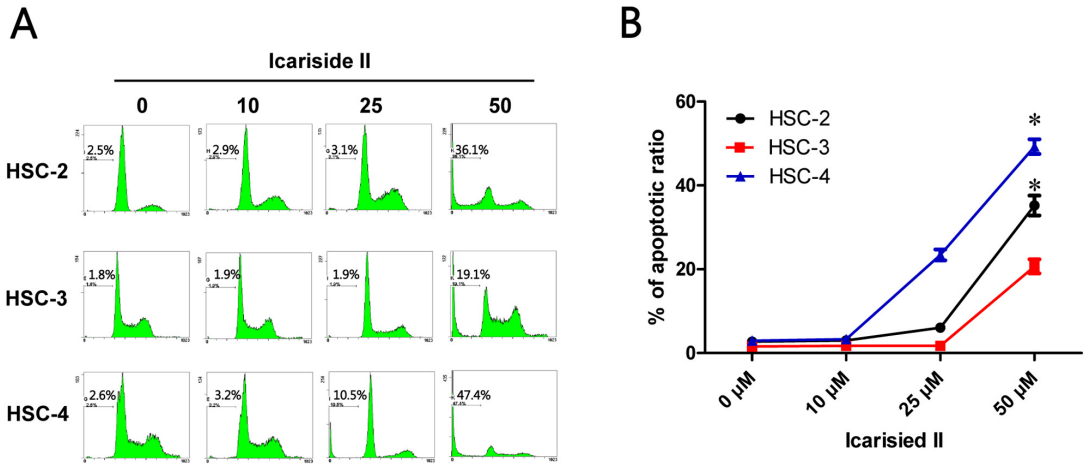

Fig. 3. Icariside II increased apoptotic cell death in the OSCC cells. The cells (HSC-2, -3, -4) were treated with Icariside II (10, 25, and 50 μM) for 24 h. (A) and (B): The ratio of apoptotic cells was determined by the FACS. Each value represents the mean of three independent experiments ± SD (n=3). *p<0.05 when compared with the control group.

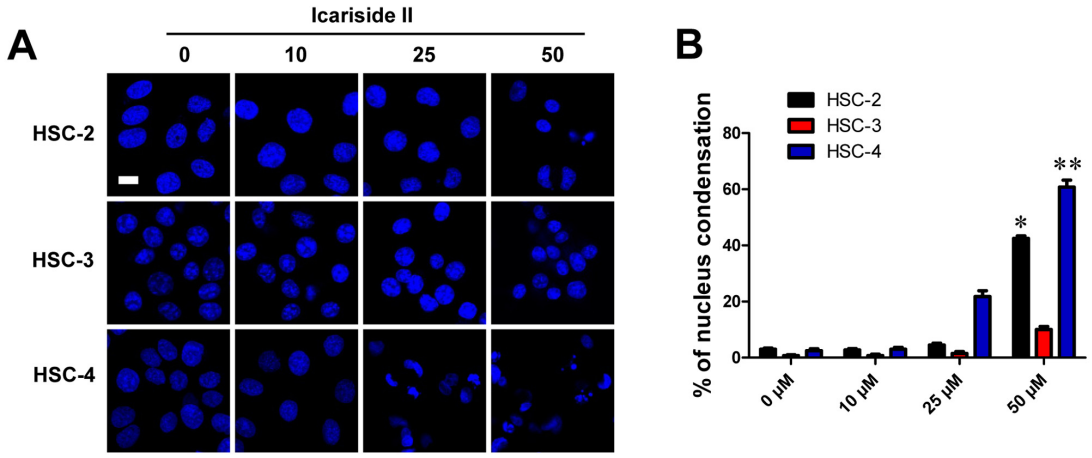

Fig. 4. The icariside II promoted morphological change of the nuclei of the OSCC cells. The cells (HSC-2, -3, and -4) were treated with icariside II (10, 25, and 50 μM) for 24 h. After treatment, the cells were stained with hoechst and observed under a fluorescence microscope. The HSC-4 cells showed a larger amount of cell shrinkage, volume reduction, apoptotic body formation, and cell blebbing than the other cell lines. Each value represents the mean of three independent experiments ± SD (n=3). *p<0.05, **p<0.01 when compared with the control group.

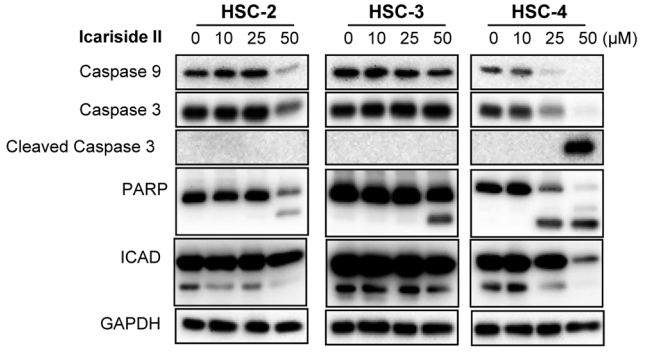

Fig. 5. The icariside II activated the apoptosis-related molecules (caspase-9, caspase-3, cleaved caspase-3, ICAD, and PARP) in the OSCC cells. The cells (HSC-2, -3, and -4) were treated with icariside II (10, 25, and 50 μM) for 24 h, and the levels of caspase-9, caspase-3, cleaved caspase-3, ICAD, and PARP were measured using western blot analysis. After the treatment, these proteins were decreased. Cleaved caspase-3 was only increased in HSC-4 cells at a level of 50 μM icariside II. The levels of GAPDH were used as an internal standard.

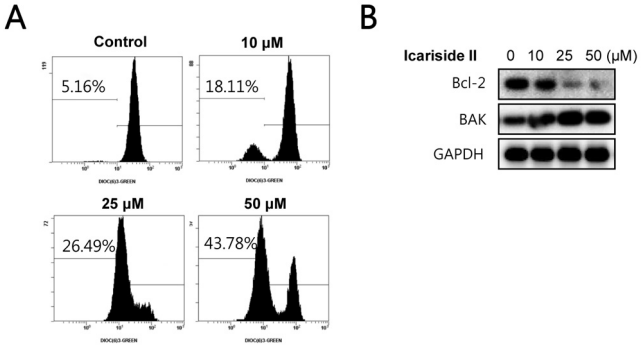

Fig. 6. Icariside II reduced the MMP and changed the expressions of the anti- and pro-apoptotic proteins. The cells (HSC-2, -3, and -4) were treated with icariside II (10, 25, and 50 μM) for 24 h. (A) The MMP (△Ψm) was reduced in a dose-dependent manner in the HSC-4 cells; (B) The pro-apoptotic molecule, Bax, was significantly increased, whereas the anti-apoptotic factor, Bcl-2, was decreased in a dose-dependent manner. The levels of GAPDH were used as an internal standard.

increased apoptotic ratio of dose dependency (Fig. 3). Hoechst staining proved that icariside II induced a change in the nuclear morphology. Untreated control cells had typical round nuclei, whereas those treated with 50 μM of icariside II for 24 h displayed condensed and fragmented nuclei in all of the OSCC cells (Fig. 4). This was especially true in the HSC-4 cells, where over 25 μM of icariside II resulted in more apoptotic cells with fragmented and cleaved nuclei (Fig. 3 and Fig. 4). To elucidate the molecular mechanisms of icariside II-induced apoptosis, the expression levels of the apoptosis-associated proteins, such as caspase-9, caspase-3, cleaved caspase-3, ICAD, and PARP, were further examined using western blot analysis. The expression levels of caspase-9, caspase-3, cleaved caspase-3, ICAD, and PARP proteins decreased at the 50 μM icariside II. In particular, cleaved caspase-3 only increased in HSC-4 cells at levels of 50 μM icariside II (Fig. 5). The results of the MMP measurement verified that a change occurred in MMP in icariside II-induced apoptosis. This occurred due to $DIOC_6$ staining, and it wasmeasured by flowcytometry. Icariside II-treated HSC-4 cells showed a loss of MMP that changed

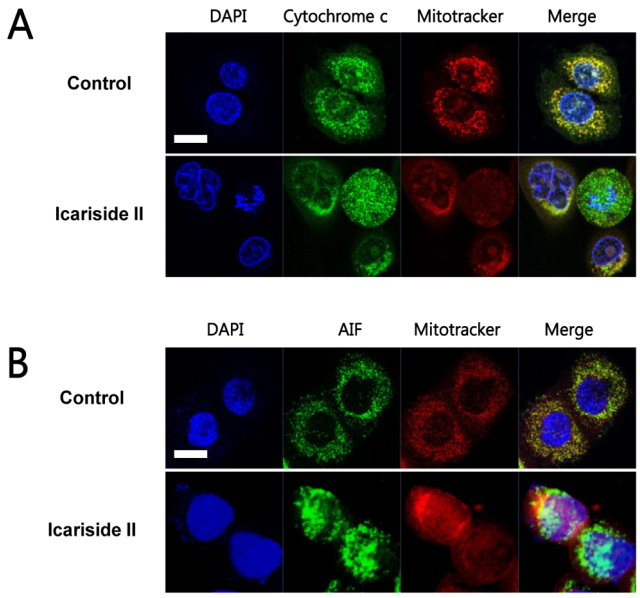

Fig. 7. The icariside II induced the translocation of the mitochondrial cytochrome c and AIF. Confocal immunofluorescence microscopy staining of the cytochrome *c* was conducted, and the AIF was in the HSC-4 cells. After the icariside II treatment: (A) cytochrome c was released from the mitochondria into the cytosol, and (B), the AIF was translocated from the mitochondria into the nuclei.

depending on the dose (Fig. 6A). The pro-apoptotic protein, Bak, was decreased, while the anti-apoptotic protein, Bcl-2, was increased by icariside II in a dose-dependent manner (Fig. 6B). Mitochondrial AIF and cytochrome c were released from the mitochondria and were detected by confocal microscopy. After icariside II treatment, cytochrome c was released from the mitochondria into the cytosol, and the AIF was translocated from the mitochondria into the nuclei (Fig. 7). These results clearly suggest that icariside II-induced apoptosis is involved in the intrinsic pathway and caspase cascades.

Discussion

Many studies have reported that plant-derived flavonoid has potent biological advantages against various types of cancer cells [21]. Icariside II has received significant attention because of its molecular biological advantages, such as its anti-inflammatory, anti-bacterial, and anti-cancer properties [22]. However, the biological mechanism behind icariside II-induced cell death against OSCC cell lines has not been well studied. OSCC is a type of malignant tumor; it is the most common oral cancer in the world. The age of onset for OSCC tends to be younger than that of other tumors (approximately 30~50 years of age) [23]. In this study, we provided emerging proof that icariin, icaritin, and icariside II have anti-cancer properties against HSC-2, -3, and -4 cells. Based on our preliminary experiments, we found that icariside II inhibits cell viability and proliferation more than icariin and icaritin in OSCC cell lines (Fig. 1). The present study investigated the effects of icariside II on the viability of HSC-2, -3, and -4 cells, revealing that icariside II produces a dose- and time-dependent reduction, particularly in the viability of HSC-4 cells. These data indicate that icariside II exerts a specific cytotoxic effect on HSC-4 cells.

During apoptosis, the cells show specific morphological and biochemical changes, including cell shrinkage, DNA fragmentation, nuclear condensation, plasma membrane blebbing, and loss of adhesion to neighboring cells or to the extracellular matrix [24, 25]. For the purpose of Hoechst staining in the present study, the cells were treated with 0 to 50 μM icariside II for 24 h, then stained with Hoechst 33324 and analyzed under a fluorescence microscope (Fig. 3). In this study, we investigated the possible mechanisms of icariside II-induced apoptosis in HSC cells. We also assessed the DNA contents that performed the flow cytometry with PI staining in the HSC cells. Treatment with icariside II resulted in an increased apoptotic ratio in HSC-2, -3, and -4 cells that was dose dependent (Fig. 4). As a result, when they were compared with the control groups, the HSC cells were consistent with the enhanced effect of icariside II on the induction of apoptosis.

In present study, we investigated the icariside II-induced apoptosis occurring via the mitochondrial signaling pathway. We found that icariside II down-regulated MMP and released mitochondrial AIF and cytochrome c from the mitochondria. A number of studies have demonstrated that flavonoids induce apoptosis through stimulation of the mitochondrial signaling pathway [18, 26, 27]. Two principal apoptosis pathways exist: the intrinsic pathway emerges from the mitochondria, while the extrinsic pathway is activated by the ligation of the death receptor [28, 29]. The intrinsic pathway leads to apoptosis under the control of the mitochondrial [30]. The cells are initiated by either extracellular stimuli or intracellular signals, after which the outer mitochondrial membranes become permeable to the internal cytochrome c. This is then released into the cytosol. Cytochrome c recruits Apaf−1 and caspase-9 to compose the apoptosome, which downstream triggers a

caspase-signaling cascade, culminating in apoptosis [31]. Thus, the anti-cancer activities of icariside II are induced by the stimulation of the mitochondrial signaling pathway.

Poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme, plays a particularly well-known role in base excision repair [32]. PARP is part of the caspase-dependent pathway of apoptosis. Cleaved PARP increases during apoptosis and is an important apoptotic marker [30, 33]. The DNA fragmentation factor, which comprises a caspase-3-activated DNase (CAD) and its inhibitor (ICAD), may influence the rate of cell death by generating PARP-activating DNA fragmentation [34, 35].

We clarified the activation of the caspases ICAD and PARP, which are associated with apoptosis by icariside II. The expression levels of caspase-9, caspase-3, ICAD, and PARP proteins activated and cleaved caspase-3, causing significant increases in the HSC-4 cells. These findings clearly suggest that icariside II-induced apoptosis via activation of the intrinsic pathway and caspase cascades in HSC-4 cell lines. Icariside II could be a potential treatment for OSCC, and it could help provide valuable data for the development of a novel anti-cancer strategy.

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Conflict of interest

The authors declare that they have no conflicting interest.

References

1. Clayman GL, Ebihara S, Terada M, Mukai K, Goepfert H. Report of the Tenth International Symposium of the Foundation for Promotion of Cancer Research: Basic and clinical research in head and neck cancer. Japanese Journal of Clinical Oncology. 1997;27:361-368. doi: 10.1093/jjco/27.5.361.
2. Casiglia J, Woo S. A comprehensive review of oral cancer. General dentistry. 2000;49:72-82.
3. Bell RB, Kademani D, Homer L, Dierks EJ, Potter BE. Tongue cancer: Is there a difference in survival compared with other subsites in the oral cavity? Journal of Oral and Maxillofacial Surgery. 2007;65:229-236. doi: 10.1016/j.joms.2005.11.094.
4. Lo WL, Kao SY, Chi LY, Wong YK, Chang RCS. Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: Factors affecting survival. Journal of Oral and Maxillofacial Surgery. 2003;61:751-758. doi: 10.1016/S0278-2391(03)00149-6.
5. Shintani S, Li CN, Mihara M, Klosek SK, Terakado N, Hino S, Hamakawa H. Anti-tumor effect of radiation response by combined treatment with angiogenesis inhibitor, TNP-470, in oral squamous cell carcinoma. Oral Oncology. 2006;42:66-72. doi: 10.1016/j.oraloncology.2005.06.010.
6. Sarvothaman S, Undi RB, Pasupuleti SR, Gutti U, Gutti RK. Apoptosis: role in myeloid cell development. Blood Res. 2015;50:73-79. doi: 10.5045/br.2015.50.2.73.
7. Tedesco I, Russo M, Bilotto S, Spagnuolo C, Scognamiglio A, Palumbo R, Nappo A, Iacomino G, Moio L, Russo GL. Dealcoholated red wine induces autophagic and apoptotic cell death in an osteosarcoma cell line. Food and Chemical Toxicology. 2013;60:377-384. doi: 10.1016/j.fct.2013.07.078.
8. Hail N, Jr., Carter BZ, Konopleva M, Andreeff M. Apoptosis effector mechanisms: a requiem performed in different keys. Apoptosis. 2006;11:889-904. doi: 10.1007/s10495-006-6712-8.
9. Guo H, Cui H, Peng X, Fang J, Zuo Z, Deng J, Wang X, Wu B, Chen K, Deng J. Modulation of the PI3K/Akt Pathway and Bcl-2 Family Proteins Involved in Chicken's Tubular Apoptosis Induced by Nickel Chloride (NiCl(2)). Int J Mol Sci. 2015;16:22989-23011. doi: 10.3390/ijms160922989.
10. Ahn G, Lee W, Kim KN, Lee JH, Heo SJ, Kang N, Lee SH, Ahn CB, Jeon YJ. A sulfated polysaccharide of Ecklonia cava inhibits the growth of colon cancer cells by inducing apoptosis. EXCLI J. 2015;14:294-306. doi: 10.17179/excli 2014-676.
11. 2006;7:1006-1014.Ruan WJ, Zhou JG. Anticancer effects of Chinese herbal medicine, science or myth? Journal of Zhejiang University Science B. doi: 10.1631/jzus.2006.b1006.
12. Hertog MG, Feskens EJ, Kromhout D, Hollman P, Katan M. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. The Lancet. 1993; 342:1007-1011. doi: 10.1016/0140-6736(93)92876-u.
13. Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: an overview. The Scientific World Journal. 2013;2013:1-6. doi:10.1155/2013/162750.
14. Pan Y, Kong LD, Li YC, Xia X, Kung HF, Jiang FX. Icariin from Epimedium brevicornum attenuates chronic mild stress-induced behavioral and neuroendocrinological alterations in male Wistar rats. Pharmacology Biochemistry and Behavior. 2007;87:130-140. doi: 10.1016/j.pbb.2007.04.009.
15. Jian L, Haiyong Y, Yijia L. Determination of rat urinary metabolites of icariin in vivo and estrogenic activities of its metabolites on MCF-7 cells. Die Pharmazie-An International Journal of Pharmaceutical Sciences. 2005;60:120-125. doi: 10.1016/j.jpba.2004.06.021.
16. Xia Q, Xu D, Huang Z, Liu J, Wang X, Wang X, Liu S. Preparation of icariside II from icariin by enzymatic hydrolysis method. Fitoterapia. 2010;81:437-442. doi: 10.1016/j.fitote.2009.12.006.

17. Xu HB, Huang ZQ. Icariin enhances endothelial nitric-oxide synthase expression on human endothelial cells in vitro. Vascular pharmacology. 2007;47:18-24. doi: 10.1016/j.vph.2007.03.002.
18. Huang C, Chen X, Guo B, Huang W, Shen T, Sun X, Xiao P, Zhou Q. Induction of apoptosis by Icariside II through extrinsic and intrinsic signaling pathways in human breast cancer MCF7 cells. Bioscience, biotechnology, and biochemistry. 2012; 76:1322-1328. doi: 10.1271/bbb.120077.
19. Zhang DC, Liu JL, Ding YB, Xia JG, Chen GY. Icariin potentiates the antitumor activity of gemcitabine in gallbladder cancer by suppressing NF-κB. Acta Pharmacologica Sinica. 2013;34:301-308. doi: 10.1038/aps.2012.162.
20. Tong JS, Zhang QH, Huang X, Fu XQ, Qi ST, Wang YP, Hou Y, Sheng J, Sun Q-Y. Icaritin causes sustained ERK1/2 activation and induces apoptosis in human endometrial cancer cells. PLoS One. 2011;6:e16781. doi: 10.1371/journal.pone.0016781.
21. Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacology & therapeutics. 2001;90:157-177. doi: 10.1016/s0163-7258(01)00137-1.
22. Kim SH, Ahn KS, Jeong SJ, Kwon TR, Jung JH, Yun SM, Han I, Lee SG, Kim DK, Kang M, Chen CY, Lee JW, Kim SH. Janus activated kinase 2/signal transducer and activator of transcription 3 pathway mediates icariside II-induced apoptosis in U266 multiple myeloma cells. European Journal of Pharmacology. 2011;654:10-16. doi: 10.1016/j.ejphar.2010.11.032.
23. Wu JY, Yi C, Chung HR, Wang DJ, Chang WC, Lee SY, Lin CT, Yang YC, Yang WCV. Potential biomarkers in saliva for oral squamous cell carcinoma. Oral oncology. 2010; 46:226-231. doi: 10.1016/j.oraloncology.2010.01.007.
24. Oropesa Ávila M, Fernández Vega A, Garrido Maraver J, Villanueva Paz M, De Lavera I, De La Mata M, Cordero MD, Alcocer Gómez E, Delgado Pavón A, Álvarez Córdoba M. Emerging roles of apoptotic microtubules during the execution phase of apoptosis. Cytoskeleton. 2015;72: 435-446. doi: 10.1002/cm.21254.
25. Nishida K, Yamaguchi O, Otsu K. Crosstalk between autophagy and apoptosis in heart disease. Circulation Research. 2008;103:343-351. doi: 10.1161/circresaha.108.175448.
26. Kim SH, Ahn KS, Jeong SJ, Kwon TR, Jung JH, Yun SM, Han I, Lee SG, Kim DK, Kang M. Janus activated kinase 2/signal transducer and activator of transcription 3 pathway mediates icariside II-induced apoptosis in U266 multiple myeloma cells. European journal of pharmacology. 2011;654:10-16. doi: 10.1016/j.ejphar.2010.11.032.
27. Lee KS, Lee HJ, Ahn KS, Kim S-H, Nam D, Kim DK, Choi DY, Ahn KS, Lu J, Kim SH. Cyclooxygenase-2/prostaglandin E 2 pathway mediates icariside II induced apoptosis in human PC-3 prostate cancer cells. Cancer letters. 2009;280:93-100. doi: 10.1016/j.canlet.2009.02.024.
28. Harada H, Grant S. Apoptosis regulators. Reviews in clinical and experimental hematology. 2003;7:117-138.
29. Wajant H. The Fas signaling pathway: more than a paradigm. Science. 2002;296:1635-1636. doi: 10.1126/science.1071553.
30. Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, Bao JK. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell proliferation. 2012;45:487-498. doi: 10.1111/j.1365-2184.2012.00845.x.
31. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA: A Cancer Journal for Clinicians. 2005;55:178-194. doi: 10.3322/canjclin.55.3.178.
32. Wall DM, McCormick BA. Bacterial secreted effectors and caspase 3 interactions. Cellular microbiology. 2014;16: 1746-1756. doi: 10.1111/cmi.12368.
33. Morales J, Li L, Fattah FJ, Dong Y, Bey EA, Patel M, Gao J, Boothman DA. Review of poly (ADP-ribose) polymerase (PARP) mechanisms of action and rationale for targeting in cancer and other diseases. Critical Reviews™ in Eukaryotic Gene Expression. 2014;24:15-28. doi: 10.1615/ critreveukaryotgeneexpr.2013006875.
34. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007;35:495-516. doi: 10.1080/01926230701320337.
35. Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H. Degradation of chromosomal DNA during apoptosis. Cell Death & Differentiation. 2003;10:108-116. doi: 10.1038/sj.cdd.4401161.