jdhs
Journal of Dental Hygiene Science

J Dent Hyg Sci Vol. 19, No. 3, 2019, pp.198-204
https://doi.org/10.17135/jdhs.2019.19.3.198

RESEARCH ARTICLE

# Protective Effects of Ursolic Acid on Osteoblastic Differentiation via Activation of IER3/Nrf2

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**Background:** Oxidative stress is a known to be associated with in the pathogenesis of many inflammatory diseases, including periodontitis. Ursolic acid is a pentacyclic triterpenoid with has antimicrobial, antioxidative, and anticancer properties. However, the role of ursolic acid in the regulating of osteogenesis remains undetermined. This study was aimed to elucidate the crucial osteogenic effects of ursolic acid and its ability to inhibit oxidative stress by targeting the immediate early response 3 (IER3)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway.

**Methods:** Cell proliferation was determined using water-soluble tetrazolium salt assay, cell differentiation was evaluated by alkaline phosphatase (ALP) activity, and formation of calcium nodules was detected using alizarin red S stain. Generation of reactive oxygen species (ROS) was determined using by DCFH-DA fluorescence dye in hydrogen peroxide ($H_2O_2$)-treated MG-63 cells. Expression levels of IER3, Nrf2, and heme oxygenase-1 (HO-1) were analyzed using western blot analysis.

**Results:** Our results showed that ursolic acid up-regulated the proliferation of osteoblasts without any cytotoxic effects, and promoted ALP activity and mineralization. $H_2O_2$-induced ROS generation was found to be significantly inhibited on treatment with ursolic acid. Furthermore, in $H_2O_2$-treated cells, the expression of the early response genes: IER3, Nrf2, and Nrf2-related phase II enzyme (HO-1) was enhanced in the presence of ursolic acid.

**Conclusion:** The key findings of the present study elucidate the protective effects of ursolic acid against oxidative stress conditions in osteoblasts via the IER3/Nrf2 pathway. Thus, ursolic acid may be developed as a preventative and therapeutic agent for mineral homeostasis and inflammatory diseases caused due to oxidative injury.

**Key Words:** Immediate early response 3 protein, NF-E2-related factor 2, Osteoblast, Oxidative stress, Periodontal disease

## Introduction

Oxidative stress is well-known for disturbing the equilibrium between the production of reactive oxygen species (ROS) and the ability of the biological system to promote antioxidant defenses[1]. ROS affect all biomolecules such as carbohydrates, membrane lipids, proteins, and nucleic acids, and thus, caused detrimental events such as physiological adaptation phenomena and disruption in the regulation of intracellular signal transduction[2]. Consequently, oxidative stress has been associated with the pathology of many inflammatory diseases[3], including cancer, atherosclerosis, hypertension, rheumatoid arthritis[4], and osteoporosis[5]. In addition, induction of oxidative stress is a potential mechanism that provokes periodontal disease which affects systemic health[6].

Periodontal disease, a chronic inflammatory disease which is induced initiated by bacterial infection, degrades the supporting tissues of the teeth. In its advanced stage, periodontitis causes a severe immune reaction in the periodontal tissues leading to tooth loss in adults and potential systemic effects[7,8]. Recently, many studies have reported that ROS contributes in inducing oxidative stress and the pathogenesis of periodontal disease in humans[9,10]. Importantly, studies have shown that oxidative stress decreases the osteogenic potential of mesenchymal stem cells, which are the progenitors of osteoblasts, adipocytes, and chondrocytes[11]. Furthermore, it has been reported to

**Received:** August 6, 2019, **Revised:** August 28, 2019, **Accepted:** September 3, 2019 eISSN 2233-7679

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cause periodontitis by upregulating osteoclastic bone resorption, which degrades the connective tissue and alveolar bone supporting the teeth during inflammatory response[12]. Several evidences have suggested that oxidative stress, as a critical mediator of pathophysiological response, is a negative regulator for bone hemostasis. It has been well documented that ROS regulates various signaling pathways that are involved in bone remodeling such as Wnt/β-catenin signaling[13], mitogen-activated protein kinase signaling[14] and the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathways[15].

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is known as a natural pentacyclic triterpenoid, which has been used traditionally as a herbal medicine in Eastern Asia for treating many clinical diseases[16]. Ursolic acid is found in various fruits, medicinal herbs, and other plants. The natural compounds has been reported to possess several biological effects, including antioxidant, anti-inflammatory, and anticancer activity[17]. Despite various beneficial effects reported in the clinical field, the underlying mechanisms of its action remain unknown.

Consequently, this study was performed to investigate the beneficial effects of ursolic acid and its underlying mechanisms of the action of ursolic acid against hydrogen peroxide ($H_2O_2$)-induced oxidative stress in the pathogenic conditions of periodontal disease.

## Materials and Methods

### 1. Cell culture

MG-63 osteoblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as per the recommended instructions. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin/ethylenediaminetetraacetic acid, and penicillin/streptomycin were from Gibco (Life Technologies, Grand Island, NY, USA). Cells were cultured in DMEM supplemented with 10% FBS, and penicillin/streptomycin (100 μg/ml) in a humidified atmosphere containing 5% $CO_2$ at 37°C. Further, cells were plated in 24-well culture plates at a density of $1\times10^4$ cells/well. For osteoblast differentiation, cells were treated with osteogenic medium (OS) containing 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. OS was replaced every 3 days. To induced oxidative stress, cells were supplemented with fresh medium and treated with different concentrations of $H_2O_2$ (100 μM) for 24 hours or 10 days.

### 2. Cell proliferation assays

Toxicity of ursolic acid (Sigma-Aldrich Corp., St. Louis, MO, USA) on MG-63 cells was assessed by water-soluble tetrazolium salt (WST-1) cell proliferation assays (Sigma-Aldrich Corp.). Briefly, after plating the cells in a 96-well plate ($5\times10^3$ cells/well) for 24 hours, they were treated with different concentrations of $H_2O_2$ (100 μM). Then, WST-1 was added each day for 7 days (10 μL/per well) and 4 hours in the dark humid conditions. Microplates were gently mixed by shaking for 1 minute and absorbance was measured at 450 nm using the Bio-Rad 680 plate-reader (Bio-Rad Labs, Hercules, CA, USA).

### 3. Reactive oxygen species measurement

ROS levels were detected using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA; Beyotime, Shanghai, China) according to the manufacturer's instructions. Cells were treated with 100 μM hydrogen peroxide in the presence or absence of ursolic acid, and incubated for 1 hour. After washing with phosphate buffer saline (PBS), the cells were stained with 10 μM DCFH-DA in the dark. Further, the cells were harvested and measured by flow cytometry (BD Biosciences, San Jose, CA, USA) with an excitation at 490 nm and an emission at 530 nm. The generation of intracellular ROS was then quantified.

### 4. Alkaline phosphatase activity activity assay

For determining alkaline phosphatase activity (ALP) activity, the cells were plated in 96-well plates at $1\times10^4$ cells/well and cultured in OS for 14 days. ALP activity was analyzed by following the protocol recommended in ALP fluorometric assay kit (BioVision, Milpitas, CA, USA). Absorbance was measured at at 405 nm on a spectrophotometer.

### 5. Alizarin red S stain

Cell culture plates were washed twice with PBS and fixed at room temperature with 4% formaldehyde for 10 min. The plates were then washed gently three times with $dH_2O$, stained at room temperature with 1% Alizarin Red S (Sigma-Aldrich) for 10 minutes, and visualized using a fluorescence microscope (Olympus IX73; Olympus Corporation, Tokyo, Japan).

### 6. Western blot analysis

PRO-PREP™ (iNtRON Biotechnology, Seongnam, Korea) was used to lyse cells, and bicinchoninic acid assay (Pierce, Appleton, WI, USA) was performed to determine the protein expression levels. Total protein (30 μg per lane) was run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, and then proteins were transferred onto a polyvinylidine difluoride membrane. The membranes were blocked with 5% non-fat milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 hour, rinsed with PBS-T, and incubated with primary antibodies against (1:1,000 dilution) overnight at 4°C. Antibody for β-actin was purchased from Sigma-Aldrich, whereas antibodies for immediate early response 3 (IER3), Nrf2, and heme oxygenase-1 (HO-1) were purchased from Cell Signaling (Lake Danvers, MA, USA). Secondary antibodies (horseradish peroxidase-conjugated) were incubated for 30 minutes, blots were visualized by chemiluminescence, and exposed to X-ray film. Image-ProPlus 5.0 software (Media Cybernetics Inc., Rockville, MD, USA) was used for quantification.

### 7. Statistical analysis

Data are presented as mean±standard deviation from a minimum of three replicates. The results were statistically analyzed by paired Student's t-test and ANOVA using IBM SPSS ver. 25.0 software (IBM Corp., Armonk, NY, USA).

## Results

### 1. Ursolic acid induced cell proliferation and differentiation in osteoblasts

At first, we determined the effect of ursolic acid on cell proliferation in MG-63 cells by using the WST-1 assays. The cells were treated with ursolic acid (0.5 μM, 1 μM, 5 μM, 10 μM) for 24 hours. The results showed that ursolic acid promoted higher proliferation rates compared to the untreated controls without a cytotoxic effect (Fig. 1A). ALP has been shown to be secreted by differentiated osteoblasts, which is correlated to the early stages of osteogenic differentiation[11]. Our data showed that ursolic acid promotes the ALP activity in a dose-dependent as well as time-dependent manner for 10 days. Remarkably, we observed that 5 μM ursolic acid treatment group showed a 242.4±12.1% increase in ALP activity compared to the

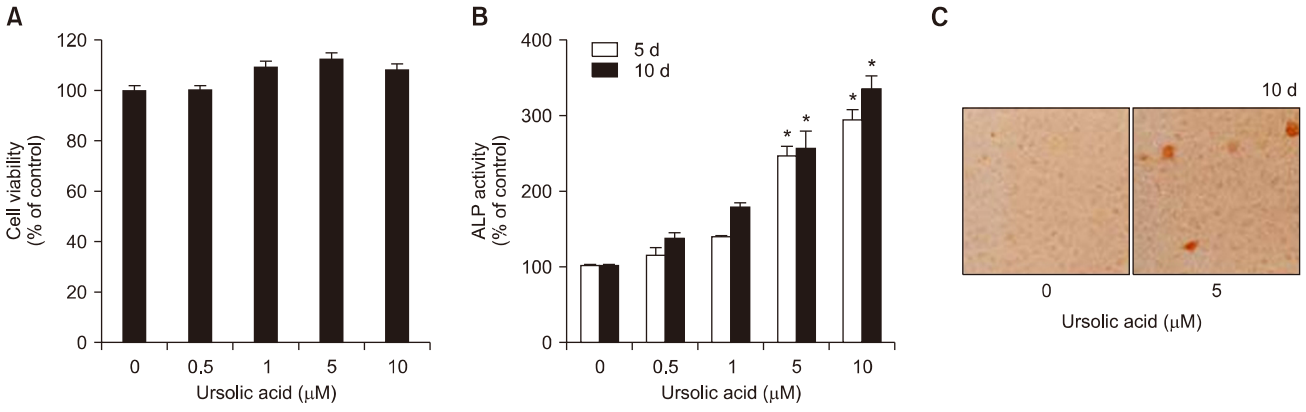


**Fig. 1.** Effects of ursolic acid on cell proliferation and differentiation of osteoblasts. (A) Cells were treated with various concentration of ursolic acid for 24 hours. Cell proliferation was evaluated by water-soluble tetrazolium salt assay. Osteoblastic differentiation was assessed by alkaline phosphatase (ALP) activity (B) and alizarin red S stain (C). Values are mean±standard deviation of three experiments. *$p < 0.05$ vs. control group.

activity shown by the untreated control group ($p<0.05$) (Fig. 1B). Furthermore, formation of mineralized nodules is one of the markers for the osteoblastic maturation. Alizarin red S staining showed that mineralized nodule formation after the osteoblasts were cultured in the OS with ursolic acid for 10 days. We found that ursolic acid up-regulates also does-dependently increase extracellular matrix mineralization in a dose-dependent manner (Fig. 1C). Thus, our results suggested that ursolic acid promotes osteogenic differentiation.

## 2. Ursolic acid inhibited $H_2O_2$-induced oxidative stress in osteoblasts

To further examine whether ursolic acid contributes in regulating oxidative stress and the differentiation in MG-63 cells during osteogenic-induction, the cells were treated with 100 μM $H_2O_2$. Many studies have suggested that the ROS generation might be a markers for oxidative stress. As shown in Fig. 2A, $H_2O_2$ treatment for 1 hour was found to markedly increased intracellular ROS by maximum of 626.1±10.2% as compared to that those of the controls ($p<0.05$). Remarkably, ursolic acid exposure was found to significantly decrease the $H_2O_2$-induced dichlorofluorescein fluorescence in dose-dependent manner ($p<0.05$) (Fig. 2B).

Furthermore, ALP activity in $H_2O_2$-treated MG-63 cells was found to be markedly decreased by 30.0% as compared to that of the control ($p<0.01$). However, we observed that the addition of 5 μM ursolic acid significantly increased the ALP activity at concentration for 10 days in $H_2O_2$-treated osteoblasts ($p<0.05$) (Fig. 2C).

## 3. Ursolic acid-mediated protective action involves immediate early response 3/nuclear factor erythroid 2-related factor 2 pathway

Accumulated evidences have showed that Nrf2 is a transcriptional factor that plays a key role in antioxidant response element-mediated induction of phase II detoxifying and antioxidant enzymes. To further investigate the molecular mechanism of ursolic acid against oxidative stress in osteoblasts, we measured the protein expression levels of IER3/Nrf2-related genes (IER3, NRF2, and HO-1) by immunoblotting. As shown in Fig. 3, the expression levels of Nrf2 and HO-1 were found to be increased in MG-63 cells when treated with ursolic acid as as compared to their expression levels in the controls and $H_2O_2$ alone-treated control cells. Conversely, the expression levels of IER3 was observed lower than the expression level in $H_2O_2$ alone-treated control cells. In addition, ursolic acid was found to up-regulate HO-1 expression in a concentration-dependent manner. Thus, our results indicated that ursolic acid has a protective effect against $H_2O_2$-induced oxidative stress by inhibiting IER3 expression, and up-regulating the expression of Nrf2 and HO-1 in osteoblasts.

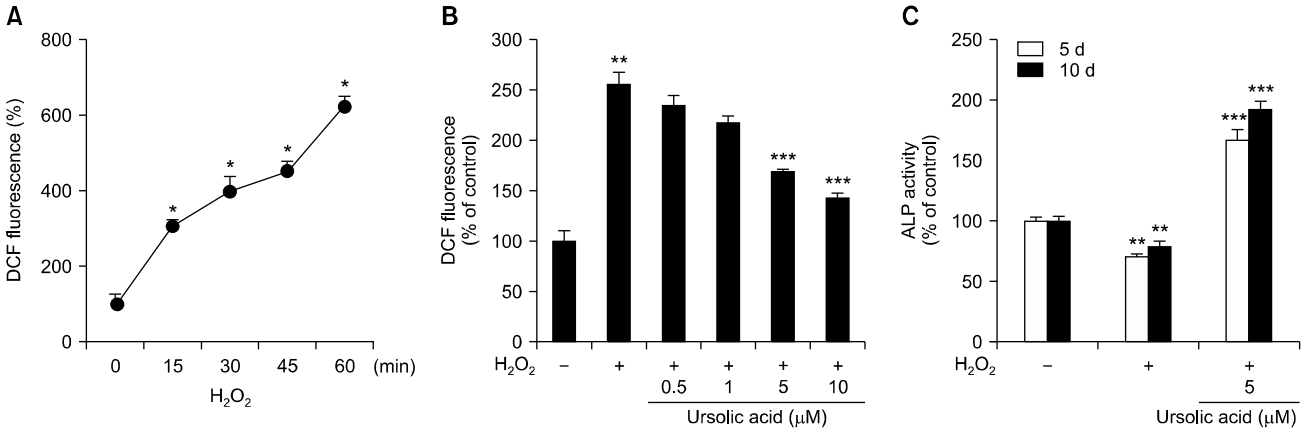


**Fig. 2.** Inhibitory effects of ursolic acid on reactive oxygen species production and osteoblastic differentiation by $H_2O_2$. (A) Cells were treated with 100 μM $H_2O_2$, and dichlorofluorescein fluorescence intensity was confirmed for 60 minutes. (B) and (C) cells were pretreated with ursolic acid (0.5~10 μM) for 2 hours and then incubated with $H_2O_2$. Values are mean±standard deviation of three experiments. *$p<0.05$ and **$p<0.01$ vs. control group, ***$p<0.05$ vs. $H_2O_2$ alone). ALP: alkaline phosphatase.

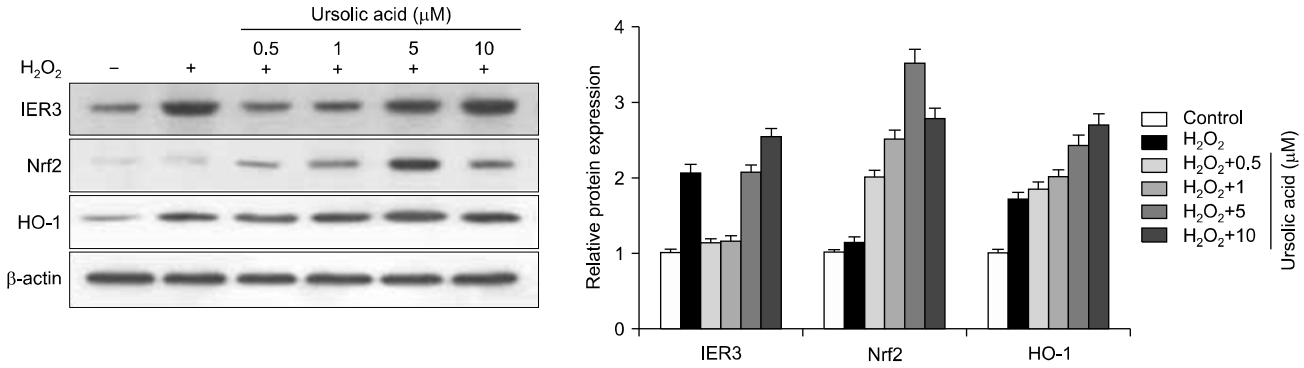


**Fig. 3.** Involvement of immediate early response 3 (IER3)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in the protective effects of ursolic acid on the $H_2O_2$-induced inhibition of osteoblastic differentiation of MG-63. Protein expression of signaling pathway-related regulators IER3, Nrf2, and heme oxygenase-1 (HO-1) were analyzed by western blot assay. The data presented are representative of three independent experiments.

## Discussion

Oxidative stress in the periodontal microenvironment is a reactive, cellular defense mechanism that produces the energy required for physiological activity, and to destroys pathogens and their components[18]. Excessive endogenous production of ROS in periodontitis leads to an imbalance in the antioxidant defense system. Increase in ROS levels in lesions might indicate that they contribute in the impairment of periodontal tissue[19]. A recent a study using dental stem cells demonstrated that oxidative stress conditions negatively influences cellular ability to repair and regenerate periodontal tissue[20].

Periodontitis is a chronic inflammatory disease that leads to bone impairment via pathogen complex and various stimulants and induces oxidative damage during chronic inflammation[9]. As imbalance in bone resorption and formation plays an important role in the disease progression, periodontal hard tissue regeneration studies have gained much attention. However, to our knowledge, there has been no definite recommended treatment for bone tissue regeneration in periodontal lesions yet.

Historically, the phytochemical and pharmacological activities of natural compounds from traditional medicines have been investigated in the early stages of drug discovery. In addition, ursolic acid has been shown to stimulate in vitro differentiation of osteoblastic cells, MC3T3-E1[21] and also proven to have in vivo bone-forming activity in a mouse calvarial bone-formation model[22]. Presently, it has been demonstrated that ursolic acid may play an important role in bone repair and regeneration.

The present study proposes that $H_2O_2$-induced production of ROS in MG-63 osteoblasts could be partially inhibited by ursolic acid. In this study, cells were treated with ursolic acid (0 μM, 0.5 μM, 1 μM, 5 μM, or 10 μM) in OS medium for 10 days to optimize the proliferation of MG-63 osteoblasts. Oure results showed that ursolic acid promoted the proliferation and osteoblastic differentiation of MG-63 cells in a dose-dependent manner. Moreover, ursolic acid was found to significantly attenuate the inhibition of osteoblastic differentiation under a high oxidative stress condition. Our results are consistent with the previous findings on stimulating osteoblast differentiation and inducing new bone formation in bone[22].

IER3, also known as immediate early gene X-1, expression is induced by a wide range of stimuli, such as growth factors, cytokines, ionizing radiation, and viral infections[23,24]. IER3 suppresses the production of ROS and protects cells from apoptosis under stress conditions. Transcription factor Nrf2, has been shown to exhibit resistance to oxidative stress and promote of bone formation[25]. Previous reports have revealed that Nrf2 regulates the expression of various proteins such as phase II detoxifying enzymes. Additionally, it has been reported that HO-1 is a downstream protein regulated by Nrf2, and associated with antioxidant genes that protect cells from various injuries via their anti-inflammatory effects and regulate disease progression[26]. Furthermore, overexpression

of NRF2 in mesenchymal stem cells is reported to enhance osteoblastic differentiation and prevent apoptosis under oxidative stress[27]. Regulation of Nrf2 activation has been reported to enhance oxidative stress during chronic inflammation by IER3. This indicate that a novel mechanism regulates Nrf2 regulation by which IER3 exhibits its stress adaptability and tumor-suppressive activity[28]. Interestingly, the current study demonstrated that ursolic acid induces protein expression of the stress response genes, *HO-1* through multifunctional stress response gene; *IER3*, and transcription factor Nrf2.

In conclusion, the present study suggests that ursolic acid promotes osteoblastic differentiation via activation of defense mechanisms. These findings provide new evidence for the potential clinical applications of ursolic acid in oxidative stress conditions associated with periodontal disease.

## Notes

### Conflict of interest

No potential conflict of interest relevant to this article was reported.

### Ethical approval

This article is not necessary for IRB screening because it is an experimental paper using commercially available cells.

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### Acknowledgements

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2018R1D1A1B07051253).

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