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Phelligridin D maintains the function of periodontal ligament cells through autophagy in glucose-induced oxidative stress

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ABSTRACT

Purpose: The objective of this study was to investigate whether phelligridin D could reduce glucose-induced oxidative stress, attenuate the resulting inflammatory response, and restore the function of human periodontal ligament cells (HPDLCs).
Methods: Primary HPDLCs were isolated from healthy human teeth and cultured. To investigate the effect of phelligridin D on glucose-induced oxidative stress, HPDLCs were treated with phelligridin D, various concentrations of glucose, and glucose oxidase. Glucose-induced oxidative stress, inflammatory molecules, osteoblast differentiation, and mineralization of the HPDLCs were measured by hydrogen peroxide (H_2O_2) generation, cellular viability, alkaline phosphatase (ALP) activity, alizarin red staining, and western blot analyses.
Results: Glucose-induced oxidative stress led to increased production of H_2O_2, with negative impacts on cellular viability, ALP activity, and calcium deposition in HPDLCs. Furthermore, HPDLCs under glucose-induced oxidative stress showed induction of inflammatory molecules (intercellular adhesion molecule-1, vascular cell adhesion protein-1, tumor necrosis factor-alpha, interleukin-1-beta) and disturbances of osteogenic differentiation (bone morphogenetic protein-2, and -7, runt-related transcription factor-2), cementogenesis (cementum protein-1), and autophagy-related molecules (autophagy related 5, light chain 3 I/II, beclin-1). Phelligridin D restored all these molecules and maintained the function of HPDLCs even under glucose-induced oxidative stress.
Conclusions: This study suggests that phelligridin D reduces the inflammation that results from glucose-induced oxidative stress and restores the function of HPDLCs (e.g., osteoblast differentiation) by upregulating autophagy.

Keywords: Cementum; Inflammation; Osteogenesis; Periodontitis

INTRODUCTION

The periodontal ligament (PDL) is a specific connective tissue that unites the alveolar bone and cementum in order to support the teeth within the jaw. In addition to its role in maintaining the teeth, the PDL also helps to supply nutrients for the repair of damaged tissue and to maintain homeostasis in periodontal tissue [1,2]. The PDL consists of mixed cell types, including fibroblasts and undifferentiated mesenchymal cells, which differentiate

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into cementoblast and osteoblast phenotypes [3,4]. Inflammation of the PDL is defined as periodontitis, which affects the supportive function of teeth [5]. Several studies have established that periodontitis is associated with diabetes mellitus (DM) [6-8]. More than three times as many DM patients have periodontitis than individuals without DM. A previous study found destruction of the periodontal tissue around the mandible and increased levels of inflammatory molecules in systemic DM-induced rats, suggesting that DM is involved in local periodontal tissue inflammation [9]. Therefore, periodontitis may be considered as a complication of DM [10].

DM is a chronic systemic disease commonly associated with neuropathy, nephropathy, retinopathy, and micro- and macro-vasculopathy [11]. The progress of diabetic complications is related to abnormal regulation of glucose levels, and DM contributes to serious periodontal diseases [12]. However, the mechanism through which DM contributes to chronic periodontitis remains unclear.

Autophagy is a natural cellular process that recycles damaged proteins and organelles in order to maintain cellular homeostasis [13]. Similarly, autophagy plays a critical role in the maintenance of cellular homeostasis by regulating cell survival and death. If the autophagy system fails to remove intracellular debris from cells, cellular apoptosis will occur, resulting in tissue damage [14]. Consequently, dysfunction in autophagy contributes to the course of various diseases, including the progression of [15]. A previous study suggested that autophagy can protect cellular homeostasis against inflammatory conditions in PDL tissue and cells [16]. However, the role of autophagy in the inflammatory action and regulation of PDL function is poorly understood in human periodontal ligament cells (HPDLCs) affected by glucose-induced oxidative stress.

Phelligridin D is a compound isolated from the *Phellinus baumii* mushroom, which belongs to the family *Hymenochaetaceae*. *P. baumii* has various biological properties, including anti-influenza, anti-oxidant, anti-inflammatory, anti-obesity, and anti-platelet effects [17-20]. It has been conventionally consumed as a food source in East Asian countries, such as Korea, Japan, and China. Previous studies have shown that phelligridin D minimizes PDL inflammation by inhibiting the mitogen-activated protein kinase pathway and restores the function of HPDLCs in lipopolysaccharide -induced periodontitis [21]. Moreover, the implantation of phelligridin D-coated titanium dental implants in rat mandible led to increased osteogenesis and osseointegration of dental implants with bone [22]. In addition, it has been reported that *P. baumii* exerts anti-diabetic activity by improving insulin sensitivity [23]. However, the effects of phelligridin D regarding the maintenance of PDL function and its relationship with autophagy have not been demonstrated yet.

In this study, we assumed that the functional and molecular alterations of HPDLCs caused by reactive oxygen species (ROS) in high-glucose conditions is similar to the cellular changes caused by DM. Therefore, the purpose of this study was to investigate the mechanism by which glucose-induced oxidative stress in HPDLCs causes dysfunction that is restored by phelligridin D.

MATERIALS AND METHODS

Materials

Alizarin red S, β-glycerophosphate disodium salt hydrate, dexamethasone, D-glucose, glucose oxidase, rapamycin (RAPA), and L-ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and interleukin-1-beta (IL-1β) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bone morphogenetic protein-2 (BMP-2), BMP-7, runt-related transcription factor-2 (RUNX-2), and β-actin were obtained from Bioworld Technology (Louis Park, MN, USA). Tumor necrosis factor-alpha (TNF-α) and light chain 3 (LC3I/II) were obtained from Cell Signaling Technologies (Beverly, MA, USA). Cementum protein 1 (CEMP-1) was purchased from LifeSpan BioSciences (Seattle, WA, USA). Autophagy related 5 (ATG5) was obtained from Novus Biologicals (Littleton, CO, USA). Beclin-1 was purchased from Bethyl Laboratories (Montgomery, TX, USA).

Isolation and identification of phelligridin D

The isolation and identification of phelligridin D followed a previously described method [24]. Briefly, ground fruiting bodies of *P. baumii* were twice extracted at room temperature with methanol. Next, the methanol was removed with reduced pressure, after which the remaining solution was divided between H_2O and n-hexane, and between H_2O and ethyl acetate. The ethyl acetate-soluble fraction was loaded into a column in Sephadex LH-20 (Amersham Bioscience, Upsala, Sweden) and eluted with methanol. Phelligridin D was purified by a Sep-pak ODS cartridge (Amersham Bioscience), and eluted with 50%–60% aqueous methanol. Phelligridin D was collected and purified by reversed-phase high-performance liquid chromatography (HPLC) (Hitachi L-2000 series, Hitachi, Tokyo, Japan) as previously reported [24].

Culture and treatment of HPDLCs with glucose oxidase to induce oxidative stress

A freshly extracted third molar was collected for culture of HPDLCs. The Human Ethics Committee of Jeonbuk National University Hospital (CUH2015-11-03) approved the protocols. HPDLCs were isolated and cultured as previously reported [21]. Briefly, PDL tissue from the third molar was isolated, minced into small pieces with a surgical scissor, and then digested with 0.5 mg/mL trypsin and 3 mg/mL collagenase type II at 37°C for 10 minutes. The pieces of tissue were cultured in a 25-mm² cell culture flask in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 300 µg/mL L-glutamine, 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37°C with 5% CO_2 in a humidified atmosphere. After the cells' growth, migrated HPDLCs from the tissue were collected and subcultured. All the experiments were completed using HPDLCs between passages 3 and 8. Mineralization of cells was performed in osteogenic media (OM) by treatment with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL ascorbic acid.

Glucose-induced oxidative stress was established following previously described methods [25]. Briefly, 80% confluent cells were supplemented with low (5 mM) and high (50 mM) D-glucose in glucose-free media and treated with 5 mU/mL glucose oxidase to induce oxidative stress for 10 days. The medium was changed with high- glucose medium and glucose oxidase every 3 days to maintain glucose-induced oxidative stress. For the detection of autophagy, the cells were treated with 1 µM of phelligridin D or 1 µM of RAPA (autophagy inducer) for the indicated time intervals.

Measurement of H$_2$O$_2$ production

The quantity of H$_2$O$_2$ production by glucose oxidase in DMEM was analyzed using a Biovision hydrogen peroxide assay kit (Biovision Research Products, Milpitas, CA, USA) according to the manufacturer's instructions at 24 hours. Absorbance was measured at a wavelength of 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-TeK, Winooski, VT, USA).

Measurement of cell viability

Cell viability was evaluated by quantifying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Following the indicated culture periods, MTT (100 μg/100 μL of phosphate-buffered saline [PBS]) solution was added to each well and incubated for 3 hours. Dimethyl sulfoxide was then added in order to dissolve the formazan crystals. Absorbance was measured at a wavelength of 570 nm with an ELISA reader (Synergy 2, Bio-TeK).

Alizarin red staining

HPDLCs were cultured in 6-well culture plates for 24 hours at 37°C. After incubation, the cells were washed with PBS and treated with 0.1 and 1 μM phelligridin D followed by glucose-induced oxidative stress. After 14 days of mineralization induction, the HPDLCs were washed with PBS, air-dried, and fixed in 95% ice-cold ethanol at −20°C for 30 minutes. These cells were stained with 40 mM alizarin red S (pH 4.2) at room temperature for 1 hour, washed 5 times extensively with deionized water, and then rinsed with PBS (without magnesium or calcium) for 15 minutes.

Alkaline phosphatase (ALP) activity

An ALP activity assay was performed in the HPDLCs after treatment with 0.1 and 1 μM of phelligridin D followed by glucose-induced oxidative stress in OM for 3, 7, and 14 days. The cells were scraped with ice-cold PBS, transferred to an ice-cold bath and sonicated with a cell disruptor in the indicated time. ALP activity was assessed in the supernatant using a SensoLyte p-NPP Alkaline Phosphatase Assay Kit (AnaSpec, Fremont, CA, USA) at 3, 7, and 10 days. The absorbance of the assay was measured using an ELISA reader (Synergy 2, Bio-TeK) at 405 nm.

Western blot analysis

A previously described method was used for western blot analysis [26]. In brief, protein samples were electroblotted onto nitrocellulose membranes after being separated by 8%–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions. These electroblotted membranes were incubated with a specified primary antibody and then a horseradish peroxidase-conjugated secondary antibody. Bands were visualized by chemiluminescent detection (Amersham Pharmacia Biotech, London, UK). Equal protein loading was confirmed in the membrane by reprobing with an antiactin antibody. Protein expression was quantified with ImageQuantT TL 1D gel program (GE Healthcare, BioScience, Sweden).

Statistical analysis

All the results were expressed as the mean±standard deviation of at least 3 independent experiments. Statistical analysis was performed using SPSS version 15.0 (SPSS, Chicago, IL, USA). Differences in the mean values for H$_2$O$_2$, cell viability, and ALP activity among cells treated with different amounts of glucose oxidase, different concentrations of glucose with or without glucose oxidase, or glucose with or without different concentration of phelligridin D were assessed by 1-way analysis of variance followed by Duncan's multiple range test. *P* values <0.05 were considered to indicate statistically significant differences.

RESULTS

Effects of glucose oxidase on H_2O_2 production, cell viability and osteogenic differentiation

HPDLCs were treated with different concentrations of glucose and glucose oxidase, and high glucose levels showed significantly increased production of H_2O_2 in a concentration-dependent manner (Figure 1A). Cell viability was detected by the MTT assay to rule out the cytotoxicity of the glucose concentration. Glucose concentrations of 25 mM and 50 mM with 5 mU/mL glucose oxidase demonstrated significantly lower cell viability than glucose levels of 5 mM and 10 mM with 5 mU/mL glucose oxidase (Figure 1B). Next, ALP activity and alizarin red staining were examined as markers of osteogenic differentiation in OM medium. ALP activity and alizarin red staining were inhibited under high-glucose conditions in the presence of glucose oxidase (Figure 1C and D).

Effects of glucose-induced oxidative stress on inflammation, osteogenic differentiation, cementogenesis, and autophagy

The higher levels of H_2O_2 production induced by high glucose levels disturbed the cell viability and osteogenic differentiation of HPDLCs. Similarly, the effects of high glucose levels on inflammation, osteogenic differentiation, and autophagy were also examined.
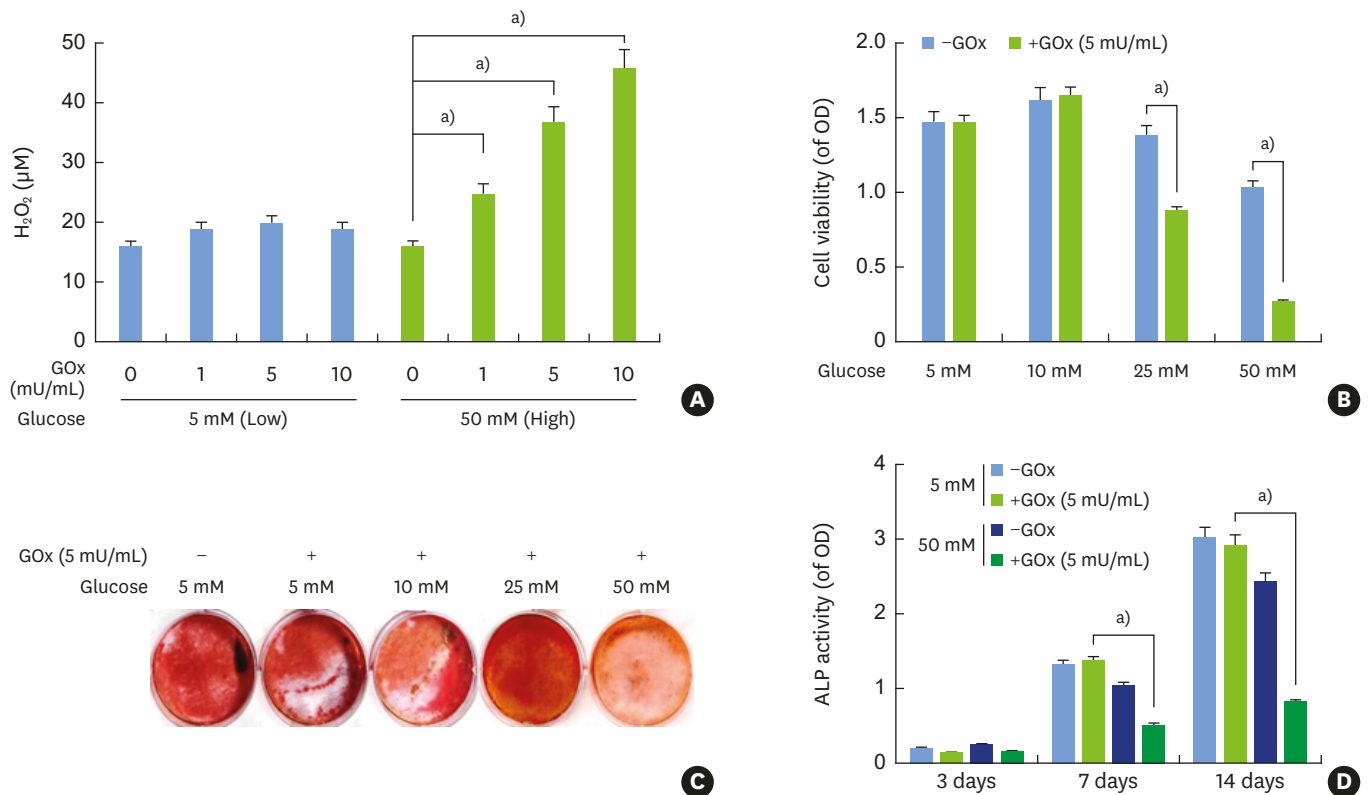

Figure 1. H_2O_2 production by GOx at various glucose levels and the relationship of H_2O_2 production with cellular toxicity and mineralization in HPDLCs. (A) Cells were stimulated with GOx (1, 5, and 10 mU/mL) in the presence of low (5 mM) or high (50 mM) glucose levels. The amount of H_2O_2 production in the culture medium was then measured at 24 hours after treatment. (B) Cell viability was determined by an MTT assay at 24 hours. (C, D) Alizarin red staining and ALP activity were analyzed as mineralization markers at the indicated interval after stimulation with GOx according to the glucose levels in HPDLCs. Each value is reported as mean and standard error of the mean of 3 independent experiments.
H_2O_2: hydrogen peroxide, HPDLC: human periodontal ligament cell, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, ALP: alkaline phosphatase, GOx: glucose oxidase, OD: optical density.
[a)]Significant difference ($P<0.05$).

Inflammatory molecules such as ICAM-1, VCAM-1, TNF-α, and IL-1β were induced by high glucose levels with glucose oxidase in comparison with low-glucose conditions (Figure 2A). However, osteogenic differentiation-related molecules (BMP-2, BMP-7, and RUNX-2), CEMP-1, and autophagy-related molecules (ATG5, LC3I/II, and beclin-1) were gradually downregulated in a time-dependent manner under high- glucose conditions in the presence of glucose oxidase (Figure 2B and C).
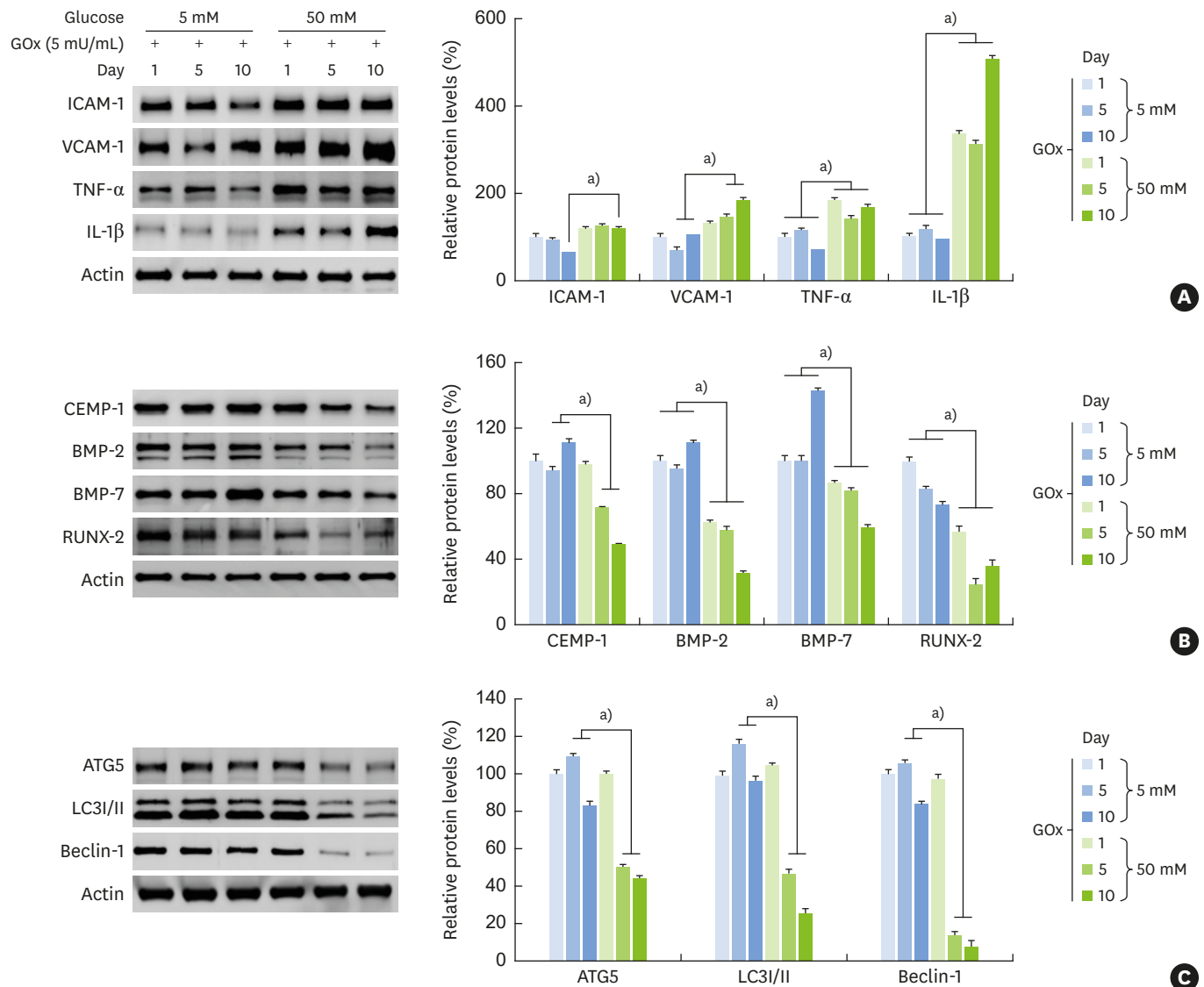
Figure 2. Influence of glucose-induced oxidative stress on inflammation, osteogenic differentiation, and autophagy. Expression of inflammatory, osteogenic differentiation-related, and autophagy-related molecules in HPDLCs under glucose-induced oxidative stress. Cells were exposed to GOx (5 mU/mL) with a low (5 mM) or high (50 mM) glucose level at intervals of 5 days and 10 days. (A) Protein expression levels of inflammatory molecules were determined by western blot analyses at the indicated times. (B) Cementogenesis and osteogenic differentiation-related markers were determined by western blot analyses. (C) Western blot analyses of autophagy-related proteins after the indicated times. All data are representative of 3 separate experiments.
HPDLC: human periodontal ligament cell, GOx: glucose oxidase, ICAM: intracellular adhesion molecule, VCAM: vascular cell adhesion molecule, TNF: tumor necrosis factor, IL: interleukin, CEMP-1: cementum protein 1, BMP: bone morphogenetic protein, RUNX-2: runt-related transcription factor-2, ATG5: autophagy related 5, LC3I/II: light chain 3.
[a)]Significant difference ($P<0.05$).

Effects of phelligridin D on cell viability and osteogenic differentiation of HPDLCs

Phelligridin D extract from *P. baumii* exhibited potent antioxidant activity. The structure and purification of phelligridin D by HPLC are demonstrated in Figure 3A. HPDLCs treated with high glucose levels and glucose oxidase showed significantly decreased cell viability in a time-dependent manner. However, treatment with phelligridin D at 0.1 or 1 μM led to the significant recovery of cell viability, even in HPDLCs that were treated with high glucose levels and glucose oxidase (Figure 3B). Alizarin red staining, as an index of bone mineralization, was also restored by treatment with phelligridin D in HPDLCs (Figure 3C). Phelligridin D also significantly restored ALP activity in HPDLCs that were treated with high glucose and glucose oxidase (Figure 3D).

Role of phelligridin D on inflammation, osteogenic differentiation, cementogenesis, and autophagy in HPDLCs under glucose-induced oxidative stress

The levels of inflammatory molecules (ICAM-1, VCAM-1, TNF-α, and IL-1β) which were increased by glucose-induced oxidative stress, were downregulated by phelligridin D in HPDLCs (Figure 4A). The anti-inflammatory properties of phelligridin D induced CEMP-1, BMP-2, BMP-7, and RUNX-2 even under glucose-induced oxidative stress in HPDLCs (Figure 4B). In addition, phelligridin D led to the recovery of ATG5, LC3I/II, and beclin-1 expression levels, which were reduced by oxidative stress (Figure 4C). Specifically, phelligridin D restored the expression of autophagy- related proteins in the same way as RAPA in HPDLCs (Figure 4D).
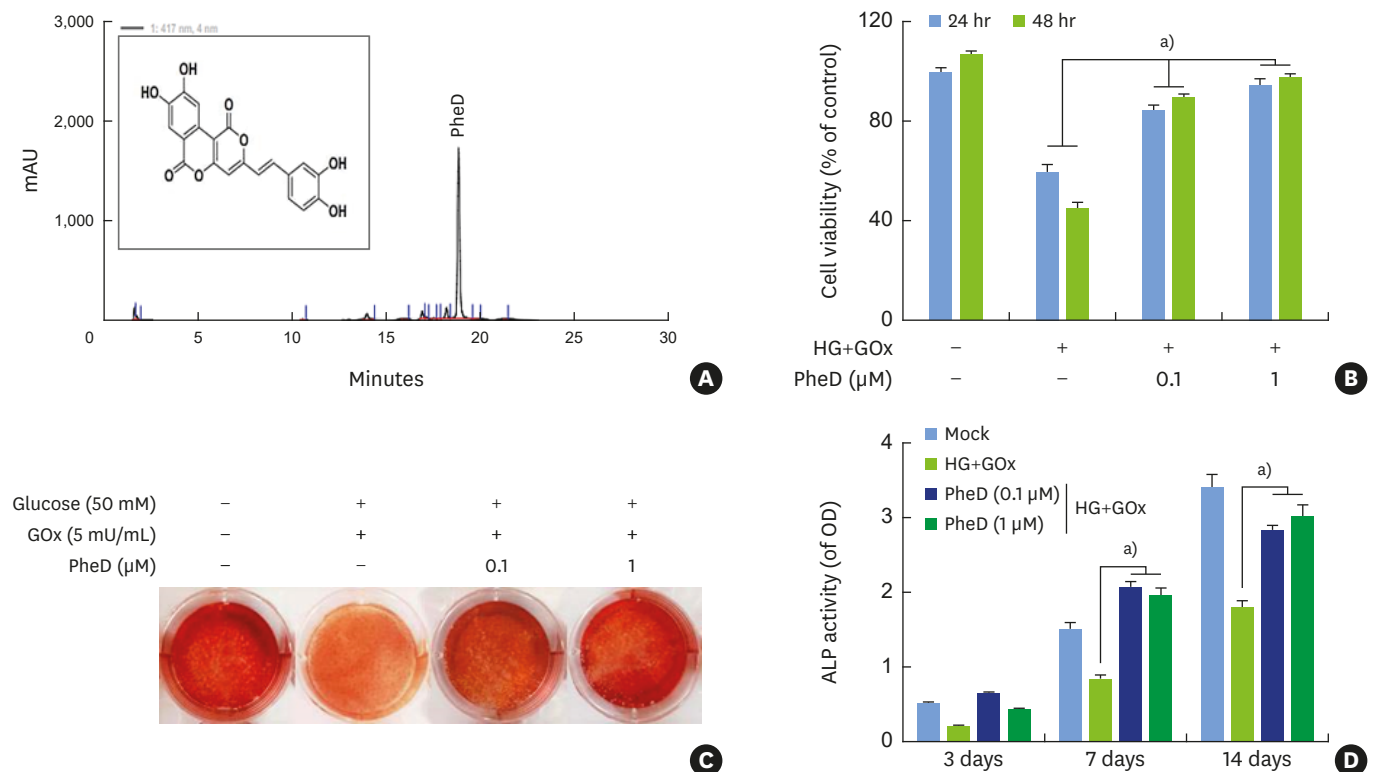
Figure 3. Effect of PheD on protection from cellular toxicity and mineralization in HPDLCs with glucose-induced oxidative stress. (A) Structure and HPLC analysis of PheD. (B) Cell viability was determined by an MTT assay at 24 and 48 hours. Mineralization assay was determined by (C) alizarin red S staining at 14 days, and (D) ALP activity at 3, 7, and 14 days. Each value is reported as the mean and standard deviation of 3 independent experiments.
HPDLC: human periodontal ligament cell, HPLC: high-performance liquid chromatography, ALP: alkaline phosphatase, HG: high glucose, GOx: glucose oxidase, PheD: phelligridin D, OD: optical density.
[a)]Significant difference ($P<0.05$).
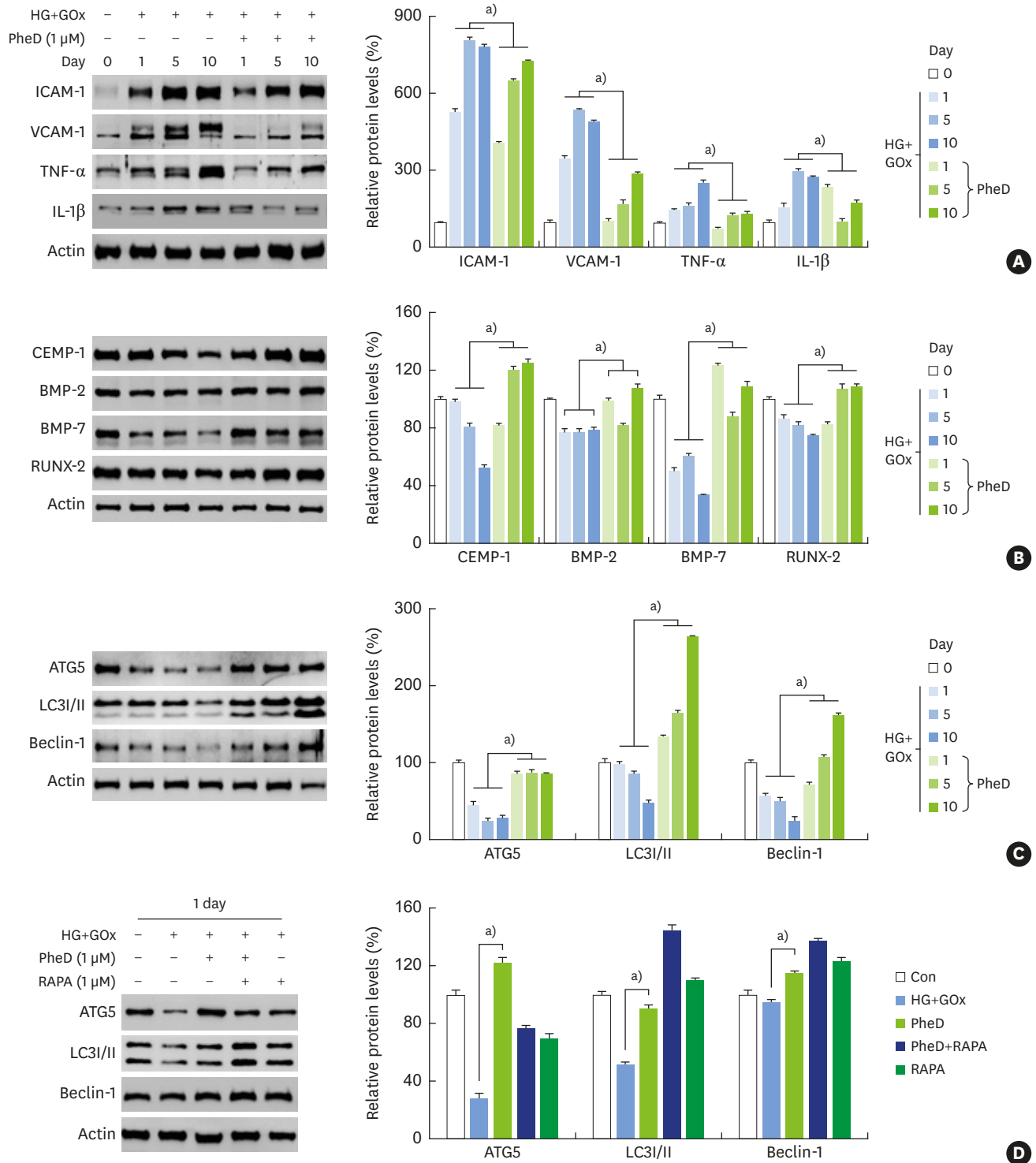
Figure 4. Effects of PheD on the expression of inflammatory, osteogenic, and differentiation and autophagy-related molecules in HPDLCs with glucose-induced oxidative stress. Protein levels were determined by western blot analyses. (A) Expression of inflammatory proteins at 1, 5, and 10 days. (B) Osteogenic differentiation-related proteins at 1, 5, and 10 days, (C) autophagy-related proteins at 1, 5, and 10 days, and (D) expression of autophagy-related proteins after treatment with PheD and RAPA (autophagy inducer) in HPDLCs with glucose-induced oxidative stress at 1 day. All data are representatives of 3 separate experiments.
HPDLC: human periodontal ligament cell, RAPA: rapamycin, ICAM: intracellular adhesion molecule, VCAM: vascular cell adhesion molecule, TNF: tumor necrosis factor, IL: interleukin, CEMP-1: cementum protein 1, BMP: bone morphogenetic protein, RUNX-2: runt-related transcription factor-2, ATG5: autophagy related 5, LC3I/II: light chain 3, HG: high glucose, GOx: glucose oxidase, PheD: phelligridin D.
[a)]Significant difference ($P<0.05$).

DISCUSSION

This study was conducted to provide basic data on the ability of phelligridin D to restore the function of PDL cells affected by periodontitis as a complication of DM. The progression of DM leads to several oral diseases, including periodontal disease, dental caries, fungal infections, and soft-tissue lesions [27]. Oxidative stress is a physio-pathological factor that has been implicated in the development of DM [28]. Under normal conditions, glucose is metabolized into D-glucono-δ-lactone and H_2O_2 by glucose oxidase. During chronic hyperglycemia, the excess production of free radicals by glucose oxidation exacerbates the complications of DM [29]. The results of this study demonstrated that exposure to glucose oxidase under high-glucose conditions resulted in high production of H_2O_2 in HPDLCs. The functional and morphological alterations of HPDLCs due to the excessive production of H_2O_2 under high-glucose conditions are similar to those observed in diabetes. H_2O_2 is a major index of oxidative stress that damages DNA, membrane lipids, and cellular proteins, and leads to cell death [30]. Therefore, treatment with glucose oxidase under high-glucose conditions induced oxidative stress similar to DM-mediated oxidative stress.

Previous studies have demonstrated that exposure to oxidative conditions increases inflammation and disturbs cellular function [31,32]. The results of this study also verified that higher production of H_2O_2 by glucose oxidase under high-glucose conditions disturbed cell viability and caused inflammation. Exposure of odontoblast or osteoblast cells to oxidative stress can disturb dental mineralization [33]. Consequently, excessive production of H_2O_2 caused HPDLCs to lose their differentiation potential to osteoblast and cementoblast phenotypes and reduced their levels of autophagy. These findings are similar to the results of previous studies [34] and suggest that high production of H_2O_2 initiates pathological conditions and impairs the vitality of the PDL.

Many studies have suggested that anti-oxidant therapies reduce the complications of DM [25,35]. Phelligridin D possesses anti-oxidant, anti-inflammatory, and anti-diabetic effects [21,36]. In our previous study, phelligridin D showed typical antioxidant effects in terms of reducing ROS activity in HPDLCs [21]. The results of this study demonstrated that the decreased cellular viability and increased expression of inflammatory molecules (ICAM-1, VCAM-1, TNF-α, IL-1β) by glucose oxidase and high glucose were restored by phelligridin D in HPDLCs, implying that the anti-inflammatory properties of phelligridin D derive from its antioxidant characteristics. Furthermore, this study showed that phelligridin D upregulated cementogenesis (CEMP-1), osteogenesis (BMP-2, BMP-7, RUNX-2), and mineralization (alizarin red, ALP activity) even under glucose-induced oxidative stress. Antioxidants can protect or restore the osteogenic differentiation and mineralization of dental pulp cells in conditions of oxidative stress [37,38]. The result of this study indicate that phelligridin D enhances osteogenic differentiation and mineralization even under glucose-induced oxidative stress in HPDLCs.

Autophagy is also involved in cellular stress, inflammation, aging, development, and cancer [39]. Autophagy maintains cellular homeostasis in response to inflammation in PDL tissue and cells [40]. In this study, glucose-induced oxidative stress decreased autophagy-related markers such as ATG5, LC3I/II, and beclin-1, whereas phelligridin D restored the expression of all these molecules even under glucose- induced oxidative stress in HPDLCs. Moreover, the finding that phelligridin D led to increased expression of autophagy-related molecules in stress induced by glucose oxidase is similar to the findings that have been observed with

RAPA, an autophagy inducer, indicating that phelligridin D promoted autophagy under conditions of glucose-induced oxidative stress. Furthermore, phelligridin D enhanced cementogenesis and osteogenic differentiation in HPDLCs.

In conclusion, this study presents new insights related to the role of phelligridin D in glucose-induced oxidative stress in HPDLCs. In these cells, phelligridin D resulted in sustained functional improvement of PDL cells with the enhancement of autophagy, subsidization of inflammation, and promotion of cementogenesis and osteogenesis. Within the limitations of this study, these findings indicate that phelligridin D may have effects making it suitable for application as a supplement to improve diabetes-related periodontal disease. In the future, additional experiments related to the detailed mechanisms and an *in vivo* study are required to demonstrate the role of phelligridin D in the treatment of DM-induced periodontal disease.

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