The Effectiveness of Asiaticoside on Osteogenic Gene Expression and Mineralization in Aging Periodontal Ligament Cells

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Abstract

The number of teeth remaining in an individual older relative to the increased risk of frailty and mortality. However, biological aging significantly affects the regenerative function of human periodontal ligament cells (hPDLC), leading to a marked decrease in osteogenic differentiation and mineralization. Asiaticoside was proven to affect the regenerative ability of hPDLCs. Therefore, this study aimed to determine the effect of asiaticoside on aging hPDLCs osteogenic gene expression and bone mineralization. The cells were retrieved from patients aged 60 and above and treated with asiaticoside at various concentrations to evaluate the percentage of cell viability using the MTT assay. Various osteogenic genes were analyzed using real-time polymerase chain reaction (PCR). Mineralization on day 14 was determined using alizarin red staining. The results show that asiaticoside had no toxicity on aging hPDLCs at concentrations of 25 µM and below. The expression of *RUNX2*, *OSX*, and *BMP9* was significantly upregulated after treatment with asiaticoside for 24 hours ($P<0.05$). Interestingly, the expression of COL1 was remarkably decreased, while *BMP2* and *DMP1* were upregulated and mineralization nodules were significantly enhanced in aging hPDLCs. This study suggested that asiaticoside has the potential to be used in periodontal regenerative treatment for elderly patients.

Keywords**:** *aging, asiaticoside, mineralization, osteogenic differentiation, periodontal ligament cell*

1. Introduction

The periodontal ligament (PDL) is a fibrous connective tissue, that adheres to the cementum and alveolar bone. The roles of PDL are to support teeth and transmit and absorb mechanical stress from occlusal loads (Shimono et al., 2003; Wills, Picton, & Davies, 1978). The PDL also provides nutrition to the cells in the periodontium through blood vessels, maintains homeostatic function, and has the intensive regenerative capability (Dean, 2017; Shimono, Hashimoto, Yamada, Abiko, & Inoue, 1988; Shimono et al., 2003). Human periodontal ligament cells show an osteoblastic phenotype. The cells can differentiate into osteoblasts, synthesize calcified nodules and produce alveolar bone (Alves et al., 2015). This regenerative ability of hPDLCs is crucial in the outcome of a periodontal treatment since it is a self-repair mechanism of periodontal organs.

However, the regenerative ability of hPDLCs is affected by age. In aging hPDLCs, the expression rates of genes involved in cell differentiation and bone remodelings such as alkaline phosphatase (*ALP*), collagen type 1 (*COL1*), and osteocalcin (*OCN*) were all downregulated compared to those expressed in young hPDLCs (Benatti, Silverio, Casati, Sallum, & Nociti, 2008; Lim, Liu, Mah, Chen, & Helms, 2014; Sawa, Yamaoka, Kuroshima, & Yoshida, 2004). As a result of the declining rate of tissue regeneration combined with long-term dental plaque and calculus accumulation, this may lead to an increased number of tooth losses in aged individuals. Hirotomi, Yoshihara, Ogawa, and Miyazaki (2015) reported an association between the number of teeth and the mortality rate in an aging population. They indicate that the more teeth remain, the higher the survival rate. Hakeem, Bernabe, and Sabbah (2019) reviewed a longitudinal study on the association between oral health and frailty. The studies showed a higher number of teeth present in the mouth and a reduction in the 3-year risk of incident frailty. In this regard, the material that can enhance periodontal regeneration in aged patients is of interest.

Asiaticoside, formed by a pentacyclic 30-carbon skeleton and glycosylate chain, is a natural product extracted from *Centella asiatica* (Figure 1). It has been used in promoting wound healing with the potential to promote collagen type 1 synthesis (Lee et al., 2006; Lu et al., 2004). In terms of periodontal regeneration, asiaticoside can promote ALP activity and significantly increase the expression of osteogenic genes such as osterix (*OSX*), dental matrix protein 1 (*DMP1*), bone sialoprotein (*BSP*), and *WNT3a*. Moreover, mineral deposition also increased when hPDLCs were treated with asiaticoside (Fitri, Pavasant, Chamni, & Sumrejkanchanakij, 2018; Nowwarote, Osathanon, Jitjaturunt, Manopattanasoontorn, & Pavasant, 2013). The results suggest that asiaticoside may be used as a potent osteoinduction material. Therefore, this study was performed to investigate the effect of asiaticoside on osteogenic induction in aging hPDLCs.

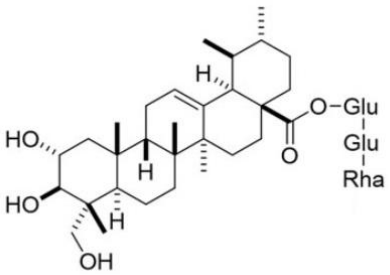

Figure 1 Asiaticoside configuration

2. Objectives

To determine the expression of osteogenesis-related genes and mineralization nodule capability in aging hPDLCs upon asiaticoside *in vitro* treatment.

3. Materials and Methods

3.1 Aging hPDLCs isolation and culture

From April 2020 to December 2020, teeth with healthy periodontium seen in radiographic images were recruited from patients aged 60 and above, according to the dental treatment plan. Periodontal ligament tissue was scraped from the middle third of the root surface, and the cells were explanted according to a previous protocol(Nowwarote et al., 2013). After explantation, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 5 mg/ml amphotericin B (Gibco, Carlsbad, CA) in an 85% humidified incubator under at 37°C and 5% CO_2. The medium was replaced every 2 days until confluence was reached. Each of the experiments was performed in triplicate using cells in passages 4-7. All experimental procedures and biosafety studies were approved by the Human Research Ethics (HREC-DCU 2020-033) and Institute Biosafety Committee, Faculty of Dentistry, Chulalongkorn University (DENT CU-IBC 009/2020).

3.2 Viability test using MTT assay

Cell viability was analyzed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; USB Corp., Cleveland, OH, USA) assay. Briefly, aging hPDLCs were seeded and treated with asiaticoside (Sigma-Aldrich, St Louis, MO) at concentrations of 12.5, 25, and 50 µM in the cultured medium for 72 hours. The control was cells incubated with dimethylsulfoxide (DMSO). Then, aging hPDLCs were washed with phosphate-buffered saline and incubated with MTT solution for approximately 30 minutes at 37°C. The formazan crystals were eluted with 1:9 DMSO/glycine buffer, including the

control. The absorbance will be measured at 570 nm using a microplate reader (Elx800; Biotek, Winooski, VT). The percentage of cell viability compared with controls will be calculated.

3.3 *Osteogenic gene expression using a real-time polymerase chain reaction*

Aging hPDLCs were seeded in 24-well plates and maintained in an osteogenic induction medium [growth medium supplemented with 10 mM β-glycerolphosphate and 50 µg/mL ascorbic acid]. The cells were treated with asiaticoside (2.5 µM and 25 µM) for 1 and 7 days. Total cellular RNA was extracted using TRIZOL® reagent (Thermo Fisher Scientific, Waltham, MA). RNA samples were converted to cDNA using a reverse transcriptase kit (ImProm-II Reverse Transcription System, Promega, USA). Real-time PCR was performed to detect target genes using an SYBR green detection system with 18S serving as an internal control (Fast Start Essential DNA Green Master; Roche Diagnostic, Indianapolis, IN). Oligonucleotide primers of the specific genes were as followed: *ALP* Forward 5' CGAGATACAAGCACTCCCACTTC and reverse 5' CTGTTCAGCTCGTACTGCATGTC; Bone morphogenic protein 2 (*BMP2*) forward 5' GCGTGAAAAGAGAGACTG and reverse 5' CCATTGAAAGAGCGTCCA C; Bone morphogenic protein 9 (*BMP9*) forward 5' CCTGGGCACAACAAGGAC and reverse 5' CCTTCCCTGGCAGTTGAG; *COL1* forward 5' CTGGCAAAGAAGGCGGCAAA and reverse 5' CTCACCACGATCACCACTCT; *DMP1* forward 5' ATGCCTATCACAACAAACCAA and reverse 5' CTCCTTTATGTGACAACTGC; *OCN* forward 5' TGACGAGTTGGCTGACCA and reverse 5' CCTTCCCTGGCAGTTGAG; *OSX* forward 5' GCCAGAAGCTGTGAAACCTC and reverse 5' GCTGCAAGCTCTGCATAACC; Runt- related transcription factor 2 (*RUNX2*) forward 5' CAGACCAGCAGCACTCCATA and reverse 5' CAGCGTCAACACCATCATTC; 18S forward 5' GGCGTCCCCCAACTTCTTA and reverse 5' GGGCATCACAGACCTGTTATT

3.4 Analyzed mineralized nodule formation using alizarin red staining

Aging hPDLCs maintained in osteogenic induction medium for 14 days will be stained with 1% Alizarin red S solution (Sigma-Aldrich, St Louis, MO). Calcium nodules were quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich, St Louis, MO) in 10 mM sodium phosphate. The optical density was measured at 570 nm compared with the control.

3.5 Statistical analysis

The data from three independent donors were statistically analyzed by a statistical software program (SPSS Version 22, Chicago, IL). The Kruskal-Wallis test was performed to determine the difference between groups. If Kruskal-Wallis was significant, Dunn's test was used to confirm a particular difference between pairs of means. All illustrations and statistical analyses were performed using GraphPad Prism version 9.2.0 (GraphPad Software, CA, USA)

4. Results and Discussion

Aging hPDLC is biologically different from young hPDLC. Evidence has shown the decreased rate of cell growth and proliferation of aging hPDLC compared with young hPDLC (Abiko, Shimizu, Yamaguchi, Suzuki, & Takiguchi, 1998; Benatti et al., 2008). Inflammatory mediators are altered, and older cells have a higher susceptibility to inflammation than young cells (Benatti, Silverio, Casati, Sallum, & Nociti, 2009). With these conditions, including the low generative ability of aging hPDLCs, periodontal treatment is challenging. In the rise of nonchemical material use trends, there are several studies about the effects of plants on cell differentiation and mineralization (Costa et al., 2016); however, the study of plants on older cells is novel.

To test the cytotoxicity of asiaticoside on aging hPDLCs, after incubating aging hPDLCs with asiaticoside for 3 days, the MTT result showed that asiaticoside did not affect the viability of cells at 12.5 and 25 µM concentrations (Figure 2). The microscopic morphology of the cells remained unchanged. At a concentration of 50 µM, the cell viability was reduced significantly ($P<0.05$) and showed shrinkage and scattered morphology under microscopic analysis (Figure 3). This result is in contrast with those of

previous studies. Fitri et al. (2018) showed that the viability of hPDLCs treated with 50 and 100 µM remained unchanged. Nowwarote et al. (2013) noted that the number of hPDLCs was decreased after incubation in asiaticoside at doses greater than 100 µg/mL. The contrast in the results may indicate that aging hPDLCs are more vulnerable than young hPDLCs. With the result of the MTT assay, asiaticoside at 2.5 and 25 µM were selected for subsequent experiments.

To determine the effectiveness of asiaticoside on osteogenic differentiation and mineralization, several osteogenic genes were analyzed using real-time PCR. After treating aging hPDLCs with asiaticoside for 1 day, the results showed that *RUNX2*, *OSX* and *BMP9* were significantly upregulated (P<0.05) when incubated with 2.5 µM asiaticoside (Figure 4). On day 7, the levels of *BMP2* and *DMP1* were significantly enhanced (P<0.05) by asiaticoside (Figure 5), whereas the level of *COL1* was significantly downregulated. *ALP* and *OCN* expression levels were not influenced by asiaticoside on either day 1 or 7. On day 14, the cells were examined for *in vitro* mineralization using alizarin red staining. The results showed that mineralization nodules were increased at all levels of asiaticoside, but only at the 25 µM concentration reach a statistically significant level (Figure 6).

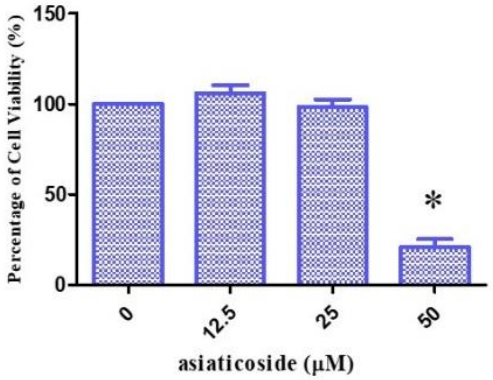

Figure 2 The cytotoxicity of asiaticoside
Aging hPDLCs were treated with 12.5, 25, and 50 µM asiaticoside for 3 days and analyzed by MTT assay. Asterisk (*) indicate statistical significance compared with the control (without asiaticoside) (P<0.05)

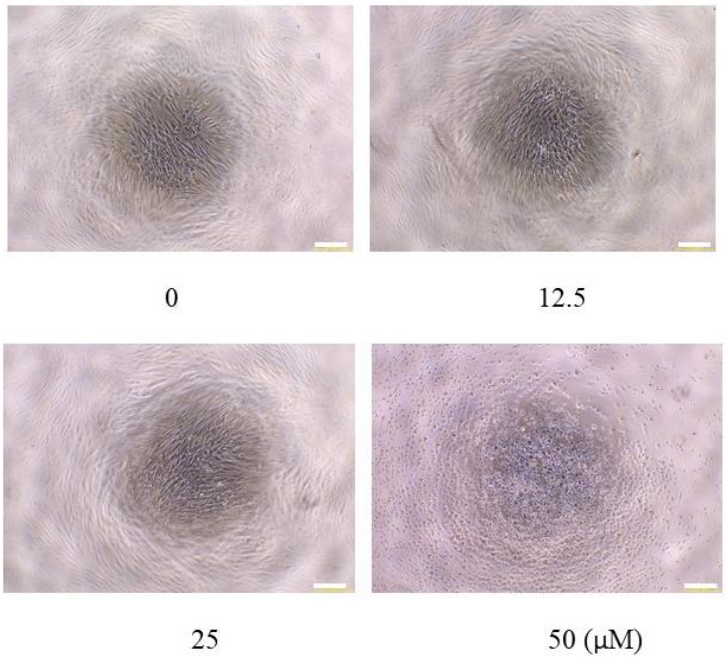

Figure 3 Aging hPDLCs morphology after treatment with 12.5, 25, and 50 µM asiaticoside for 3 days. Aging hPDLCs with 0 µM asiaticoside was used as a control. The micrograph was viewed at a magnification of 4X, scale bar = 300 µm.

Osteogenic/osteoblastic differentiation is undergoing 3 stages: proliferation (preosteoblast), matrix maturation, and matrix mineralization (Lian & Stein, 1992) with various genes involved. *RUNX2* is an early gene marker that is active in the proliferation stage of osteogenic differentiation. This gene is required for osteoblast-specific gene expressions, such as *OSX*, *OCN*, osteopontin (*OPN*), and bone sialoprotein (*BSP*), for the maturation of osteoblasts (Karsenty & Wagner, 2002; Rutkovskiy, Stensløkken, & Vaage, 2016). *OSX* is a transcription factor that functions as an inhibitor of Wnt, the pathway of *RUNX2* regulation. *OSX* can also induce the expression of essential osteogenic genes, such as *ALP*, *OCN*, and *DMP1* (Amarasekara, Kim, & Rho, 2021; Liu et al., 2020). In our study, after treating aging hPDLCs with asiaticoside for 1 day, *RUNX2* and *OSX* expression were significantly enhanced, which correlated with a previous study, showing that the upstream of *OSX* was influenced by the expression of *RUNX2* (Liu et al., 2020). However, Fitri et al. (2018) showed that OSX expression was significantly upregulated when the cells were treated with asiaticoside and that the expression of *RUNX2* was not altered, which is in contrast with our results. The variation in the results may be influenced by the aging status of the cells. There is evidence showing lower expression of *RUNX2* in aging hPDLCs than in young hPDLCs (Wu, Bi, Yu, Zhang, & Chen, 2015). This suggested that since the baseline is low, with the induction of asiaticoside, the expression of genes was remarkably enhanced.

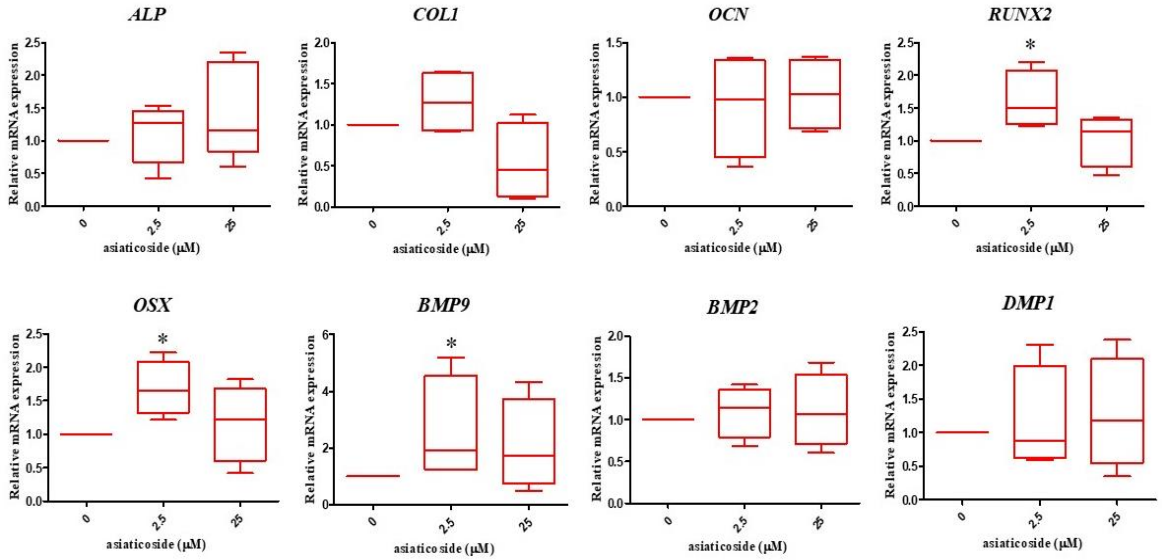

Figure 4 The influence of asiaticoside on osteogenic gene expression in aging hPDLCs
Cells were treated with asiaticoside at concentrations of 2.5 and 25 μM for 1 day. The mRNA levels of *ALP*, *COL1*, *OCN*, *RUNX2*, *OSX*, *BMP9*, *BMP2*, and *DMP1* were measured using real-time PCR. Asterisks (*) indicate statistical significance compared with the control (P<0.05)

Bone morphogenic proteins (BMPs), known as growth differentiation factor 2 (GDF2) promote osteogenic differentiation by regulating the transcription of osteoblastic genes such as *RUNX2* and *OSX* (Lamplot et al., 2013). On day 1, *BMP9* gene expression was significantly upregulated when cells were treated with 2.5 μM asiaticoside. The expression of *BMP9* was correlated with upstream *RUNX2* and *OSX* since BMPs regulate *RUNX2* transcription and work in concert with *RUNX2* to regulate the transcription of target genes (Rutkovskiy et al., 2016). In contrast, asiaticoside induced *BMP2* expression on day 7, which correlated with the expression pattern of the *BMP2* gene in hPDLCs during osteogenic differentiation (Choi, Noh, Park, Lee, & Suh, 2011). DMP1 is a noncollagenous transcription protein that functions in the early and late stages of osteogenic differentiation. DMP1 initiates osteogenic differentiation by transcription of osteoblastic genes and extracellularly regulate the nucleation of hydroxyapatite (Narayanan et al., 2003). In our study, asiaticoside significantly promoted the gene expression on day 7, which was during the matrix maturation stage. Fitri et al. (2018) showed the significant upregulation of *DMP1* after treatment with asiaticoside for 1 day, which was during the preosteoblastic stage. Nowwarote et al. (2013) presented the increased level of *DMP1* compared with the control on day 14, which was during the matrix mineralization stage, indicating that asiaticoside may influence *DMP1* expression at every stage of osteogenic differentiation. The effect of asiaticoside on early osteogenic gene markers (*BMP9*, *RUNX2* and *OSX*) and genes involved in matrix maturation and mineralization stage (*BMP2* and *DMP1*) may suggest that asiaticoside promoted every stage of osteogenic differentiation.

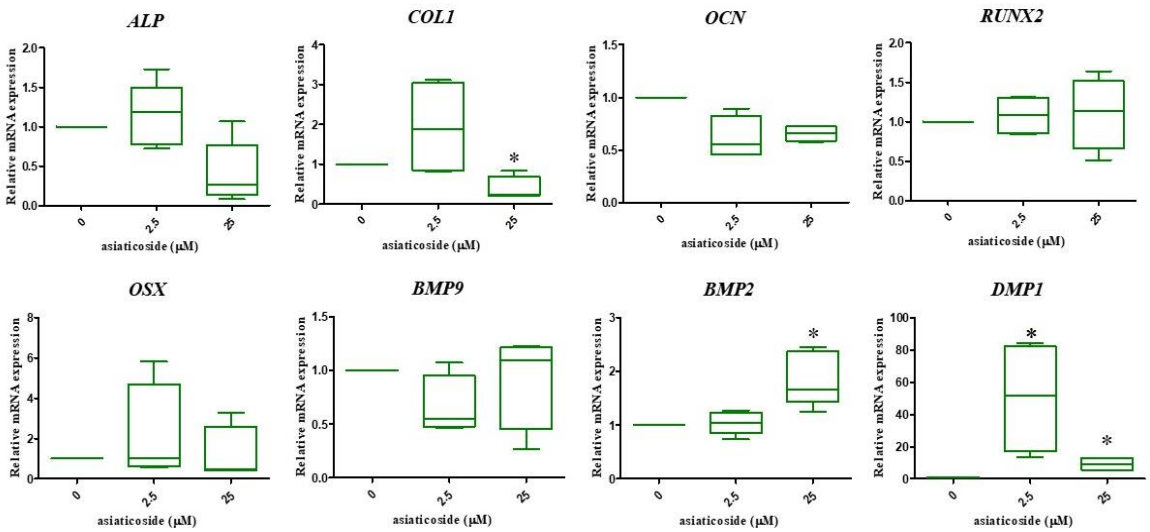

Figure 5 The influence of asiaticoside on osteogenic gene expression in aging hPDLCs.
Cells were treated with asiaticoside at concentrations of 2.5 and 25 μM for 7 days. The mRNA levels of *ALP*, *COL1*, *OCN*, *RUNX2*, *OSX*, *BMP9*, *BMP2*, and *DMP1* were measured by real-time PCR.
Asterisks (*) indicate statistical significance compared with the control (P<0.05)

The interesting result of our experiment is the expression of *COL1*. On day 7, *COL1* expression was found to be significantly downregulated at the 25 μM concentration. Asiaticoside is a well-known material that promotes collagen synthesis in human dermal cells and hPDLCs (Lee et al., 2006; Lu et al., 2004; Nowwarote et al., 2013). However, there is also evidence that asiaticoside can suppress *COL1* expression and deposition. Zhang et al. (2020) proved that asiaticoside promotes the expression of *BMP7* and Smad1/5 expression. *BMP7* promotes phosphorylated Smad1/5 complex and inhibits collagen deposition. Tang et al. (2011) found that asiaticoside has an inhibitory effect on *COL1* expression through the TGF-β/Smad signaling pathway in a dose- and time-dependent manner. These contradictory results indicate that the effect of asiaticoside on *COL1* expression can be variable.

ALP and *OCN* were found to be maximally expressed during the matrix maturation and matrix mineralization stages in hPDLC (Choi et al., 2011). ALP is a metalloenzyme, that initiates mineralization by hydrolyzing inorganic pyrophosphate (PPi) to inorganic phosphate (Pi). Pi will bind with calcium and form calcium phosphate hydroxyapatite, which is the main structure of alveolar bone and cementum (Vimalraj, 2020). OCN is a bone-specific protein, synthesized by osteoblast and is present as a noncarboxylated hormone in bone (Rubert & De la Piedra, 2020). The expression of *ALP* and *OCN* in our study was not different between asiaticoside-treated cells and the control on both day 1 and day 7. Despite the unaltered gene expression, the mineralized nodule was significantly upregulated at the same concentration. To produce hydroxyapatite, the ratio of Pi and PPi accumulation is crucial. An excessive amount of PPi will inhibit calcification (Liang, Hu, Li, & Liu, 2021). The authors hypothesized that asiaticoside can regulate the Pi and PPi ratio, which needs to be further investigated.

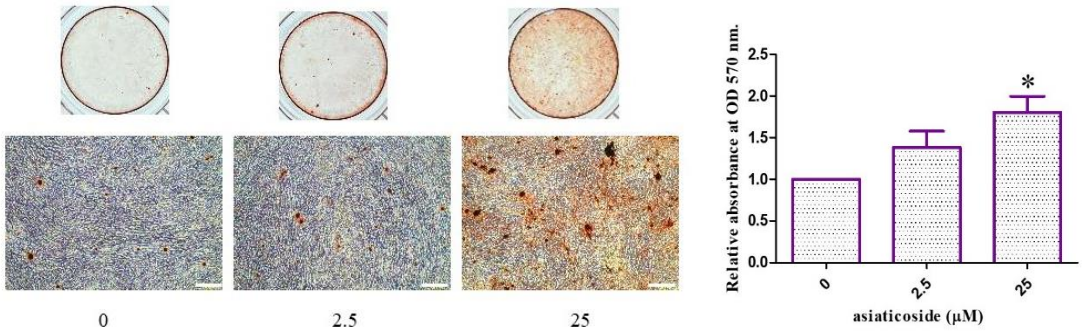

Figure 6 Mineralization was increased in aging hPLDCs after treatment with asiaticoside
Alizarin red staining was used to determine the mineralized ability of aging hPDLCs in asiaticoside for 14 days. The accumulation of calcification nodules in red colors was shown (upper left panel) and the micrograph at a magnification of 10X was shown (left lower panel); scale bar = 100 μm. The amount of deposit calcium was quantified (Right). Asterisks (*) indicate statistical significance compared with the control (P<0.05)

With the upstream *BMP2* and *DMP1* expression and the upregulation of calcifying nodules at 25 μM asiaticoside, this may suggest that *DMP1* and *BMP2* play important roles in promoting mineralization in aging hPDLCs treated with asiaticoside. However, the mechanism of action is unknown. Asiaitocoside may activate the BMP/Smad signaling pathway as in a report of previous studies (Lee et al., 2006; Zhang et al., 2020) or upstream the number of DMP1 binding calcium ions, transported extracellularly and was phosphorylated to produce hydroxyaptatite (Narayanan et al., 2003). Asiaticoside was also proven to affect several osteogenic pathways, including the Wnt pathway and TGF-β Smad signaling (Fitri et al., 2018; Lee et al., 2006). The variation in the results may indicate that asiaticoside not only promoted every stage of osteogenic differentiation but also affected several osteogenic pathways.

Periodontitis is one of the most prevalent chronic conditions in the aging population (Eke et al., 2016). Severe periodontitis can lead to tooth loss. Poor masticatory function due to tooth loss leads to sarcopenia and musculoskeletal dysfunction and a decline in physical performance with the development of frailty (Iwasaki et al., 2018). To prevent this incidence, hard and soft periodontal tissue regeneration is needed. The present study is the first to investigate the effect of asiaticoside on aging hPDLCs, and the results indicated that asiaticoside may be used as a regenerative material in treating periodontitis in an aging population. Nevertheless, the effect of asiaticoside needs to be confirmed in an aging animal model or other *in vivo* experiments. Further studies are required to identify the mechanism of action in osteogenic differentiation and mineralization to develop the usage of asiaticoside in clinical application.

5. Conclusion

Asiaticoside can enhance osteogenic gene expression and induce mineralization. It has the potential to be used as an alternative periodontal regenerative material in clinical applications.

6. Acknowledgments

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