EUROPEAN ENDODONTIC JOURNAL

ORIGINAL ARTICLE

Effects of D-galactose Induction on Aging Characteristics of the Human Dental Pulp Cell Culture Model: An *In Vitro* Study

Suthasinee SAIYASILP,[1] Savitri VASEENON,[2] Tanida SRISUWAN,[2] Patchanee CHUVEERA[1]

[1]Division of General Dentistry, Department of Family and Community Dentistry, Chiang Mai University, Faculty of Dentistry, Chiang Mai, Thailand

[2]Department of Restorative Dentistry and Periodontology, Chiang Mai University, Faculty of Dentistry, Chiang Mai, Thailand

ABSTRACT

Objective: This study aimed to investigate the effects of D-galactose (D-gal) on cellular senescence induction, cell proliferation, mineralization production, and odontogenic gene expression of isolated human dental pulp cells (HDPCs).

Methods: Isolated HDPCs were cultured and assigned to four groups: control, 1 g/L D-gal, 10 g/L D-gal, and 10 g/L D-gal with Biodentine (BD). Cell proliferation was evaluated at 24, 48, and 72 hours using Alamar Blue® assay. To evaluate cellular senescence at 48 hours, senescence-associated beta-galactosidase (SA-β-gal) activity and senescence-related genes (p16 and p21) were assessed with SA-β-gal staining assay and quantitative reverse-transcription polymerase chain reaction (qRT-PCR), respectively. To examine the mineralization potential under differentiating conditions, quantitative staining with Alizarin Red S and mineralization-related gene expression (dentine sialophosphoprotein, DSPP) were investigated at 14 days. One-way ANOVA was used for statistical analysis. The statistical significance level was set at 0.05.

Results: 1 g/L D-gal and 10 g/L of D-gal significantly decreased cell proliferation at 72 hours compared to the control group ($p<0.05$). SA-β-gal-positive cells were significantly more prevalent in both D-gal-treated groups than in the control group ($p<0.05$). The expressions of genes p16 and p21 were markedly increased in cells treated with 10 g/L D-gal compared to the control group ($p<0.05$). The addition of BD did not promote cell proliferation but significantly improved cellular senescence by reducing SA-β-gal activity, p16, and p21 expression ($p<0.05$) compared to the group without BD. For mineralization potential, the amount of mineralization was similar among groups under differentiating conditions. The reduction of DSPP gene expression was obvious only in the 10 g/L D-gal group ($p<0.05$). The addition of BD did not show a significant effect on mineralization.

Conclusion: Ten g/L of D-gal can effectively induce aging phenotypes and reduce DSPP gene expression in HDPCs. Co-incubation with BD extract reduced the expression of these aging phenotypes. Mineralization production was not altered in the presence of D-gal. The data support the development of *in vitro* model for aging dental pulp.

Keywords: Biodentine, cellular senescence, dental pulp cells, D-galactose, SA-β-gal

HIGHLIGHTS

- Ten g/L of D-galactose can promote cellular senescence in the human dental pulp cells.
- D-galactose shows a potential to use as an aging accelerator in an *in vitro* vital pulp therapy model under aging situation.
- Biodentine has an anti-aging property in dental pulp cells.

Please cite this article as:
Saiyasilp S, Vaseenon S, Srisuwan T, Chuveera P. Effects of D-galactose Induction on Aging Characteristics of the Human Dental Pulp Cell Culture Model: An *In Vitro* Study. Eur Endod J 2025; 10: 142-150

Address for correspondence:
Patchanee Chuveera
Division of General Dentistry, Department of Family and Community Dentistry, Chiang Mai University, Faculty of Dentistry, Chiang Mai, Thailand
E-mail: patchanee.ch@cmu.ac.th

Received : July 31, 2024,
Revised : October 01, 2024,
Accepted : November 15, 2024

Published online: March 18, 2025
DOI 10.14744/eej.2024.15010

INTRODUCTION

Age-related alterations occur in all tissues and organs, including teeth (1) where the function and physiological activities of the dental pulp decline, which limit its responses to external stimuli. Cellular senescence is one of the key markers observed during aging conditions. It involves an irreversible arrest of cell division

caused by various stress inducers, such as infection, inflammation, and oxidative stress, which induce irreparable DNA damage, triggering cell cycle arrest while secreting a variety of substances into the environment (2). Cellular senescence is a concern in regenerative dentistry since it could impair the regeneration process of the dental pulp (3).

At present, treatment options for injured dental pulp have gradually shifted from conventional to more regenerative approaches, such as vital pulp therapy (VPT) and regenerative endodontic procedures (REP) (4). Initially applied in young patients, these treatments have later been adopted for older patients as well (5). Although the clinical success of VPT using bioactive materials such as mineral trioxide aggregate (MTA) or Biodentine (BD) is favorable, few studies have been conducted on cell regeneration in aged dental pulp cells. Therefore, developing an *in vitro* aging model of dental pulp cells for future research is of great importance.

There are various models that have been used to explore the underlying mechanisms of aged dental pulp, including harvesting healthy dental pulp from aged patients (6) or animal models (7), and treating isolated dental pulp cells with certain chemical substances (8). A number of approaches, which include the observation of the senescence-associated beta-galactosidase (SA-β-gal) lysosomal enzyme and the detection of biomarker genes for cellular senescence, such as p16 and p21, have been used to examine the mechanism behind the aging process (9). Previous studies have revealed a decline in pulp function in dental pulp isolated from aged patients (6, 10). Comparable results have also been described in some *in vitro* studies that simulate the aging model in dental pulp cells using hydrogen peroxide (8, 11) or para-Cresol (p-Cresol) (12).

To date, one of the most well-known substances used to induce cellular aging in many types of organs and tissues, both *in vitro* and *in vivo*, is D-galactose (D-gal), which is an aldohexose-reducing sugar that exists in the body and is abundantly found in most foods (13). Overdosing on exogenous D-gal causes aging by increasing oxidative stress, apoptosis, and inflammation (14). Several studies have used D-gal to induce the aging process in many organs, such as the brain, heart, lungs, liver, kidneys, reproductive organs, skin, bones, skeletal muscles, immune response, and others (15). The D-gal-induced aging model is generally applied in research because of its practicality, short study duration, ease of application, lower cost, minimal adverse effects, and because it is less likely to cause death in animals in *in vivo* models (16). However, to the best of our knowledge, D-gal has never been studied in dental pulp cells.

Accordingly, this study aimed to explore the effects of D-gal on isolated human dental pulp cells (HDPCs) in various aspects associated with cellular senescence, including cell proliferation, SA-β-gal expression, p16 and p21 gene expression, mineralization potential, and the expression of dentine sialophosphoprotein (DSPP) genes. Additionally, the effects of BD, a common bioactive material used for VPT, on D-gal-treated HDPCs were also examined. The null hypothesis of this study posited that D-gal did not induce cellular senescence in HDPCs, nor did BD supplementation alleviate the senescence condition.

MATERIALS AND METHODS

Primary Culture of Human Dental Pulp Cells (HDPCs)

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was endorsed by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University (no: 83/2020, date: 25/11/2020). Human dental pulp tissues were aseptically harvested from sound third molars (n=3) from patients aged 18–25 years without any underlying disease. After tooth extraction, blood and saliva were rinsed off from the extracted teeth using sterile normal saline solution. The tooth crown was then immersed in 2.5% sodium hypochlorite (NaOCl) for 5 minutes for disinfection. The teeth were sectioned into halves with a sterilized chisel and hammer. The pulp tissues were hygienically separated from the sectioned teeth in a laminar flow hood using sterilized forceps, then ground and digested in a solution containing Collagenase I (Gibco/Invitrogen, Gaithersburg, MD, USA) and Dispase II (Sigma-Aldrich) for up to 45 minutes. Then the samples were centrifuged at 1,500 rpm at 25°C for 5 minutes to eliminate the undigested tissue. The cell suspension was transferred and cultured in complete α-MEM (Sigma-Aldrich, St Louis, MO, USA) consisting of 10% fetal bovine serum (Gibco/Invitrogen), 1% penicillin-streptomycin (Sigma-Aldrich), and 100 mol/L L-ascorbic acids (Sigma-Aldrich) at 37°C humidified atmosphere of 95% air and 5% CO_2 with medium refilled every 72 hours. Then, the cells were subcultured with 0.25% trypsin-EDTA (Gibco/Invitrogen). To determine cell proliferation and senescence biomarkers, HDPCs at the third passage were assigned to 4 experimental groups as follows: 1) control: cells cultured in complete α-MEM; 2) 1 g/L D-gal: cells cultured in complete α-MEM with 1 g/L D-gal; 3) 10 g/L D-gal: cells cultured in complete α-MEM with 10 g/L D-gal; and 4) 10 g/L D-gal with BD: cells cultured in complete α-MEM with 10 g/L D-gal and BD extract. To analyze mineralization potential and the expression of dentinogenic differentiation gene, HDPCs at the third passage were assigned to 5 experimental groups as follows: 1) control: cells cultured in complete α-MEM throughout the experiment; 2) control with differentiation induction: cells cultured in complete α-MEM for 48 hours, then in differentiation media for another 14 days; 3) 1 g/L D-gal: cells cultured in complete α-MEM with 1 g/L D-gal for 48 hours, then in differentiation media for 14 days; 4) 10 g/L D-gal: cells cultured in complete α-MEM with 10 g/L D-gal for 48 hours, then in differentiation media for 14 days; and 5) 10 g/L D-gal with BD: cells cultured in complete α-MEM with 10 g/L D-gal and BD extract for 48 hours, then in differentiation media for 14 days.

Preparation of D-galactose (D-gal) and Biodentine (BD)

The procedure and concentration for D-gal preparation were modified from previous studies. In brief, D-gal powder (Sigma-Aldrich) was dissolved in α-MEM medium and filtered through a 0.2 μm filtration membrane (Minisart; Sartoris Stedim Biotech, Gõettingen, Germany) to prepare the D-gal solution of 1 and 10 g/L. A volume of 100 mL each was prepared for the 1 and 10 g/L D-gal solutions.

The preparation of BD extract was modified as described by Weekate et al., (17). Briefly, after mixing 1 capsule of BD

(Septodont, Saint-Maurdes-Fossés, France) following the manufacturer's instruction, it was loaded into 10 cylindrical elastic molds (2 mm in width and 3 mm in height) and incubated at 37°C for 24 hours to allow the BD to set. A total of 10 BD pieces were prepared, which was sufficient for all experiments. Each BD piece was then immersed in 3.14 mL of sterile α-MEM for 7 consecutive days. The supernatant was then filtered using a 0.2 µm membrane and kept at 4°C. The BD extracts were diluted in complete α-MEM to a ratio of 1:16 prior to the experiment, and a total volume of 502.24 mL of diluted BD extract was prepared.

Cell Proliferation by Alamar Blue® Assay

Cells seeded at $1x10^3$ cells/well in 96-well plates were allocated to groups as described earlier. For each sample (n=3), cells were assigned in triplicates per group. At 24, 48, and 72 hours, cell proliferation was evaluated using the Alamar Blue® assay (Gibco/ Invitrogen). Briefly, 15 µL of Alamar Blue were added into each well, and then the quantitative changes in colorimetric were evaluated using a spectrophotometer (Bio-Rad Laboratories Inc., Herculus, CA, USA) at the wavelength of 550 and 595 nm. The percent reduction of Alamar Blue was calculated.

Analysis of Senescence Biomarkers

Senescence-associated beta-galactosidase (SA-β-gal) assay

HDPCs at $1×10^4$ cells/well were seeded into 24-well plates and assigned similarly to the experimental groups in the previous part. For each sample (n=3), cells were assigned in triplicates per group. At 48 hours, SA-β-gal staining was carried out using SA-β-gal staining kit (Cell Signaling Technology, Beverly, MA, USA) in accordance with the manufacturer's instructions. In short, after fixing the cells in 2% (v/v) formaldehyde and 0.2% glutaraldehyde, the cells were then kept in an SA-β-gal staining suspension (1 mg/mL 5-bromo-4-chloro-3-indolyl h-D-galactosidase, 40 mM citric acid, pH 6.0, 40 mM sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide (K4[Fe(CN)6]), 5 mM potassium ferricyanide (K3[Fe(CN)6]), 150 mM sodium chloride, and 2 mM magnesium chloride) and later incubated at 37 °C without CO2 for 24 hours. The senescent positive cells were identified as blue-staining by standard light microscopy. Ten random fields per well were used to identify the proportion of SA-β-gal-positive cells using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Senescence-related Gene Expression by Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)

Cells at $3×10^5$ cells/well in 6-well plates were used in this part. Cells from each sample (n=3) were assigned in triplicates per group. At 48 hours, total RNA was separated with the NucleoSpin RNA II extraction kit (ThermoFisher Scientific) and transcribed into cDNA using ReverTra Ace qPCR RT master mix kit (Toyobo Co. Ltd, Osaka, Japan). The genes upregulation levels were explored by quantitative real-time polymerase chain reaction assay in triplicate using LightCycler 480 Real-Time PCR system (Roche Applied Science, Rotkreuz, Switzerland) with SensiFAST SYBR no-ROX kit (Bioline, London, UK). The PCR cycles, temperatures, and time were set as following: 1) denaturation: 45 cycles, 95°C, 10 seconds; 2) annealing: 60°C, 20 seconds; and 3) extension: 72°C, 40 seconds. The primer sequences for p16 and p21 were designed following the published literature shown in Table 1. The amount of each mRNA was standardized by the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) and calibrated using the comparative CT method.

TABLE 1. Primer sequences used for the detection of senescence markers by qRT-PCR

Gene name	Primer sequences (5'-3')	
p16	Forward: CAA CGC ACC GAA TAG TTA CG Reverse: GAA GGA CCT GTG CGA CCA	(32)
p21	Forward: GTC TTG TAC CCT TGT GCC TC Reverse: AAA GAT GGT GAG GTT TGC TG	(32)
GADPH	Forward: ACC ACA GTC CAT GCC ATC AC Reverse: TCC ACC ACC CTG TTG CTG TA	(32)

qRT-PCR: Quantitative reverse-transcription polymerase chain reaction, GADPH: Glyceraldehyde-3-phosphate dehydrogenase

Analysis of Mineralization Potential and the Expression of Dentinogenic Differentiation Gene

Alizarin red S assay

HDPCs at $3×10^3$ cells/ in 24-well plates were assigned to 5 groups as mentioned earlier in the primary culture of HDPCs section. Cells from each sample (n=3) were assigned in triplicates per group. Differentiation media, consisting of complete α-MEM, 50 mg/mL of ascorbic acid (Sigma-Aldrich), 10 nmol/mL of dexamethasone (Sigma-Aldrich), and 10 mmol/mL of β-glycerophosphate (Sigma-Aldrich), was used for the following groups: differentiation media, 1 g/L D-gal, 10 g/L D-gal, and 10 g/L D-gal with BD; except for the control group. At day 14, samples were fixed with 4% paraformaldehyde and rinsed with 1 mL of phosphate-buffered saline (PBS). Then, 0.5 mL of Alizarin Red S solution at pH of 4.2 (Sigma-Aldrich) was added and incubated with a gentle rocking motion for 5 minutes at room temperature. The remaining dye was removed with deionized water. After that, all samples were left dry at room temperature for a week. To quantify the volume of calcium deposit, 10% methylpyridinium chloride monohydrate (Sigma-Aldrich) in 10 mmol/L sodium phosphate was used as the destaining solution (room temperature, 20 minutes). One hundred microliters of the destaining solution were relocated to 96-well plates, and the absorbance was assessed using a spectrophotometer at 550 nm.

Mineralization-related Gene Expression by Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)

Cells at $1×10^5$ cells/well in 6-well plates were treated as previously described in osteogenic differentiation media. The cells were assigned to groups in the same fashion as the Alizarin Red S assay. Cells from each sample (n=3) were assigned in triplicates per group. At day 14, total RNA was collected and processed for DSPP gene expression using qRT-PCR. The primer sequences are shown in Table 2.

TABLE 2. Primer sequences used for the detection of mineralization-related marker by qRT-PCR

Gene name	Primer sequences (5'-3')	
DSPP	Forward: AAT GGG ACT AAG GAA GCT G Reverse: AAG AAG CAT CTC CTC GGC	(32)
GADPH	Forward: ACC ACA GTC CAT GCC ATC AC Reverse: TCC ACC ACC CTG TTG CTG TA	(32)

qRT-PCR: Quantitative reverse-transcription polymerase chain reaction, DSPP: Dentine sialophosphoprotein, GADPH: Glyceraldehyde-3-phosphate dehydrogenase

Statistical Analysis

All experiments were conducted in triplicate and expressed as the mean±standard error. Statistical analysis was performed by SPSS 17.0 software (SPSS Inc, Chicago, IL, USA) using One-way ANOVA and either LSD or Dunnett's T3 test. Since the variances gathered from data of cell proliferation, mineralization formation, and the expression of odontogenic differentiation-related gene were equal, the post hoc LSD was applied. The Dunnett's T3 test was performed in results obtained from SA-β-gal expression and senescence-related gene expression as the variances were unequal. The dissimilarity between experimental groups was considered to be statistically significant at $p<0.05$.

RESULTS

Effects of D-gal on HDPC Proliferation

At 24 hours after cell treatment, no significant changes in the percentage of cell proliferation were observed among the D-gal 1 g/L (20.52±4.52%), D-gal 10 g/L (19.23±3.46%), and D-gal 10 g/L + BD groups (19.40±3.39%) when compared with the control (20.99±4.23%, $p>0.05$). Similarly, the cell proliferation at 48 hours of the D-gal 1 g/L (35.50±2.84%), D-gal 10 g/L (34.31±2.21%), and D-gal 10 g/L + BD groups (34.99±2.42%) did not differ significantly when compared with their control (37.62±2.79%, $p>0.05$). However, D-gal at both 1 and 10 g/L significantly reduced the percentage of cell proliferation (36.05±0.53% and 35.99±0.78%, respectively) when compared to the control group at 72 hours (39.63±0.81%, $p<0.05$). The addition of BD in 10 g/L D-gal for 72 hours did not improve cell proliferation as the percentage of cell proliferation (36.81±0.99%) was not comparable to the control (39.63±0.81%, $p<0.05$). Moreover, no differences in cell proliferation between D-gal 10 g/L (35.99±0.78%), and D-gal 10 g/L + BD groups (36.81±0.99%) were observed at 72 hours ($p=0.48$) (Fig. 1).

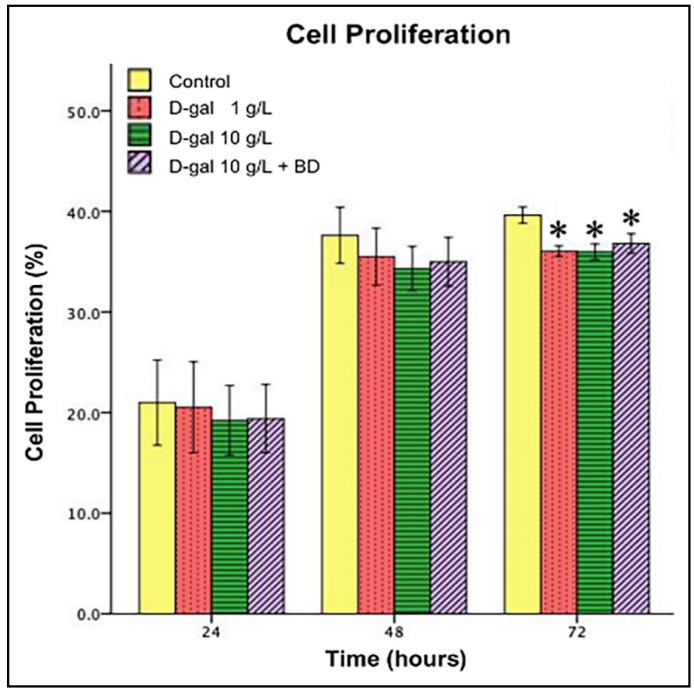

Figure 1. Cell proliferation of D-gal-induced HDPCs at different concentrations. * $p<0.05$ compared to the control group. Statistical analyses were performed by One-way ANOVA

BD: Biodentine; D-gal: D-galactose, HDPCs: Human dental pulp cells, ANOVA: Analysis of variance

Effects of D-gal on the Expression of Senescence Biomarkers

SA-β-gal expression

The detection of SA-β-gal positive cells was examined at 48 hours incubation period. SA-β-gal positive cells were stained in blue (Fig. 2a). Quantitatively, the percentage of SA-β-gal positive cells remarkably increased in all groups containing D-gal ($p<0.05$). In groups with 10 g/L D-gal showed a significantly higher number of SA-β-gal positive cells (56.06±1.69%) than in a group with 1 g/L D-gal (33.76±1.97%, $p<0.05$). Co-incubation with BD significantly lowered the number of SA-β-gal positive cells (49.59±1.07%, $p<0.05$) when compared to the 10 g/L D-gal group without BD (Fig. 2b).

Senescence-related gene expression

The expression of senescence-related genes p16 and p21 were assessed at 48 hours using qRT-PCR. Increasing trends in p16 and p21 expression were observed in both D-gal groups without BD. Considering the expression of p16 gene, the significant upregulation was detected only in the 10 g/L D-gal group (2.41±0.13-fold change) when compared to the control ($p<0.05$). Interestingly, the presence of BD significantly downregulated the level of p16 (1.32±0.05-fold change) when compared to the 10 g/L D-gal group ($p<0.05$) (Fig. 3a). BD not only significantly decreased the p16 gene expression compared to the 10 g/L D-gal group, but it also restored p16 levels to those of the control group ($p=0.06$). Similarly, a significant increase of p21 gene expression was found in the 10 g/L D-gal group (2.11±0.08-fold change) when compared to the control ($p<0.05$). Co-incubation of BD with 10 g/L D-gal significantly lowered the p21 level (0.58±0.06-fold change) when compared to the10 g/L D-gal group ($p<0.05$). The level of p21 gene in the co-incubation group was comparable with the control ($p=0.06$) as well (Fig. 3b).

Effects of D-gal on the Mineralization Potential and Odontogenic Differentiation

Mineralization formation

The Alizarin Red S assay was performed to quantify calcium deposition on day 14 under differentiating conditions (Fig. 4a). Mineral deposition was compared with the control groups. The control groups, which received no treatment, were divided

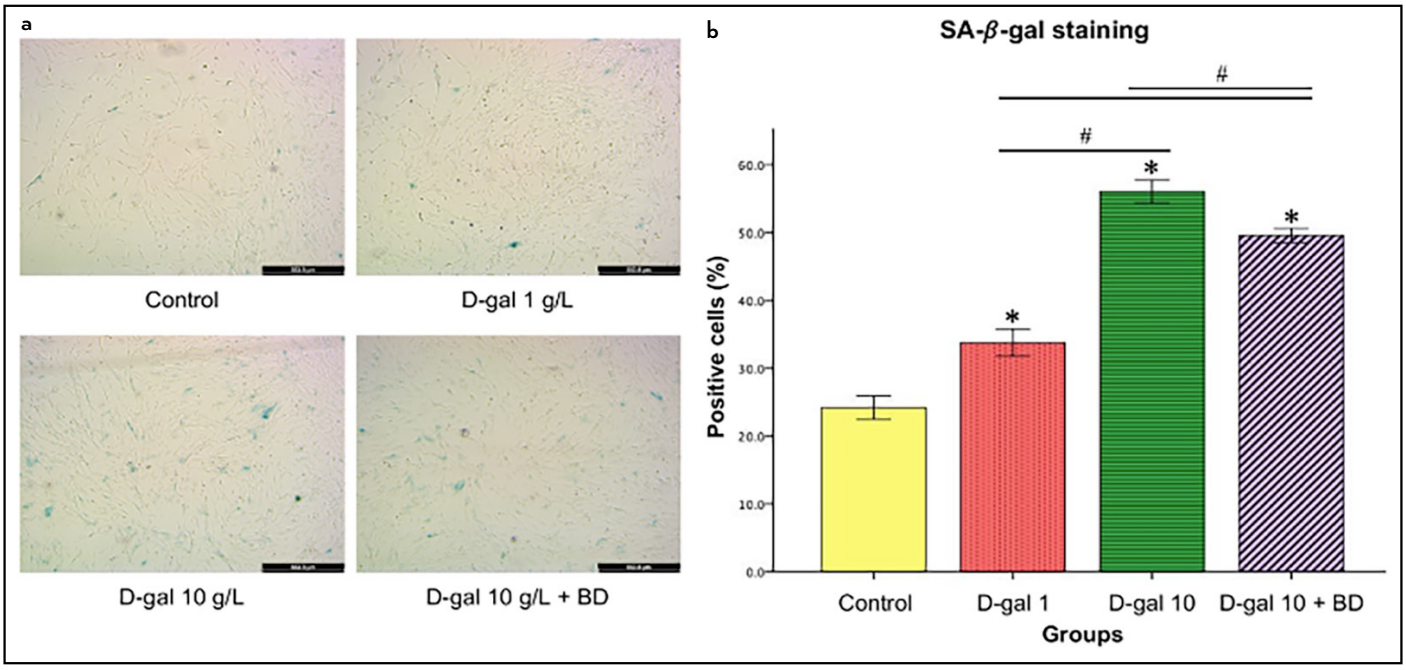

Figure 2. Effects of D-gal on the expression of senescence biomarkers. (a) SA-β-gal positive cells shown in blue, scale bar = 553.8 µm. (b) Quantification of SA-β-gal positive cells

*: p<0.05 compared to the control group, #: p<0.05 compared to other experimental groups. SA-β-gal: Senescence-associated beta-galactosidase, BD: Biodentine; D-gal: D-galactose

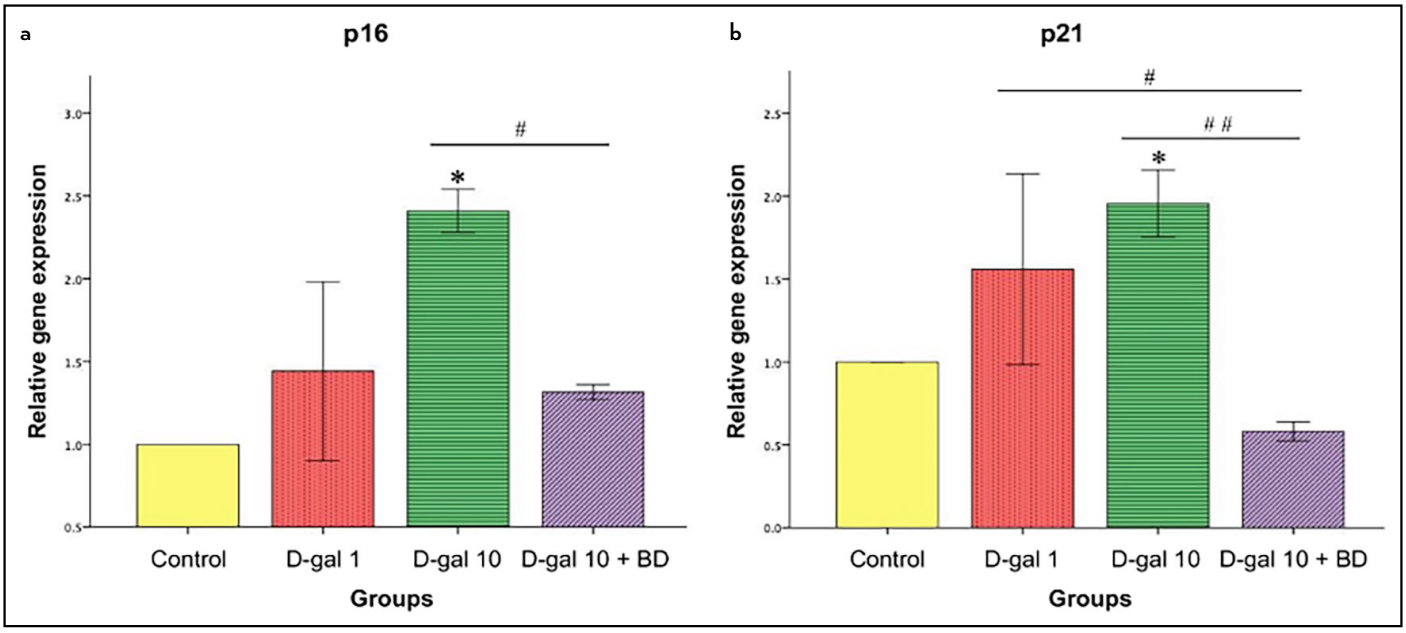

Figure 3. The effects of D-gal and co-incubation with BD on senescence-related gene expression using qRT-PCR analysis at 48 hours. (a) The expression of p16. (b) The expression of p21. GADPH was used as the internal control.

*: p<0.05 compared to the control group, #: p<0.05, ##: p<0.01 compared to the different concentration. D-gal: D-galactose, BD: Biodentine, qRT-PCR: Quantitative reverse-transcription polymerase chain reaction, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

into 2 subgroups: one without differentiation induction and the other that underwent differentiation for 14 days, as previously described in the materials and methods. All groups subjected to differentiation induction, including the second control group (1.47±0.13), 1 g/L D-gal group (1.42±0.13), 10 g/L D-gal group (1.32±0.13), and the combined 10 g/L D-gal with BD group (1.48±0.14) showed significantly higher absorbance readings, indicating increased mineralization compared to the control group without differentiation medium (0.26±0.07, p<0.05). Comparison among groups with differentiation medium revealed no significant changes, though the absorbance showed a decreasing trend when cells were exposed to D-gal, especially at higher concentrations. Co-incubation of BD with 10 g/L D-gal did not enhance mineralization as no significant differences were detected compared to the 10 g/L D-gal group (p=0.37) (Fig. 4b).

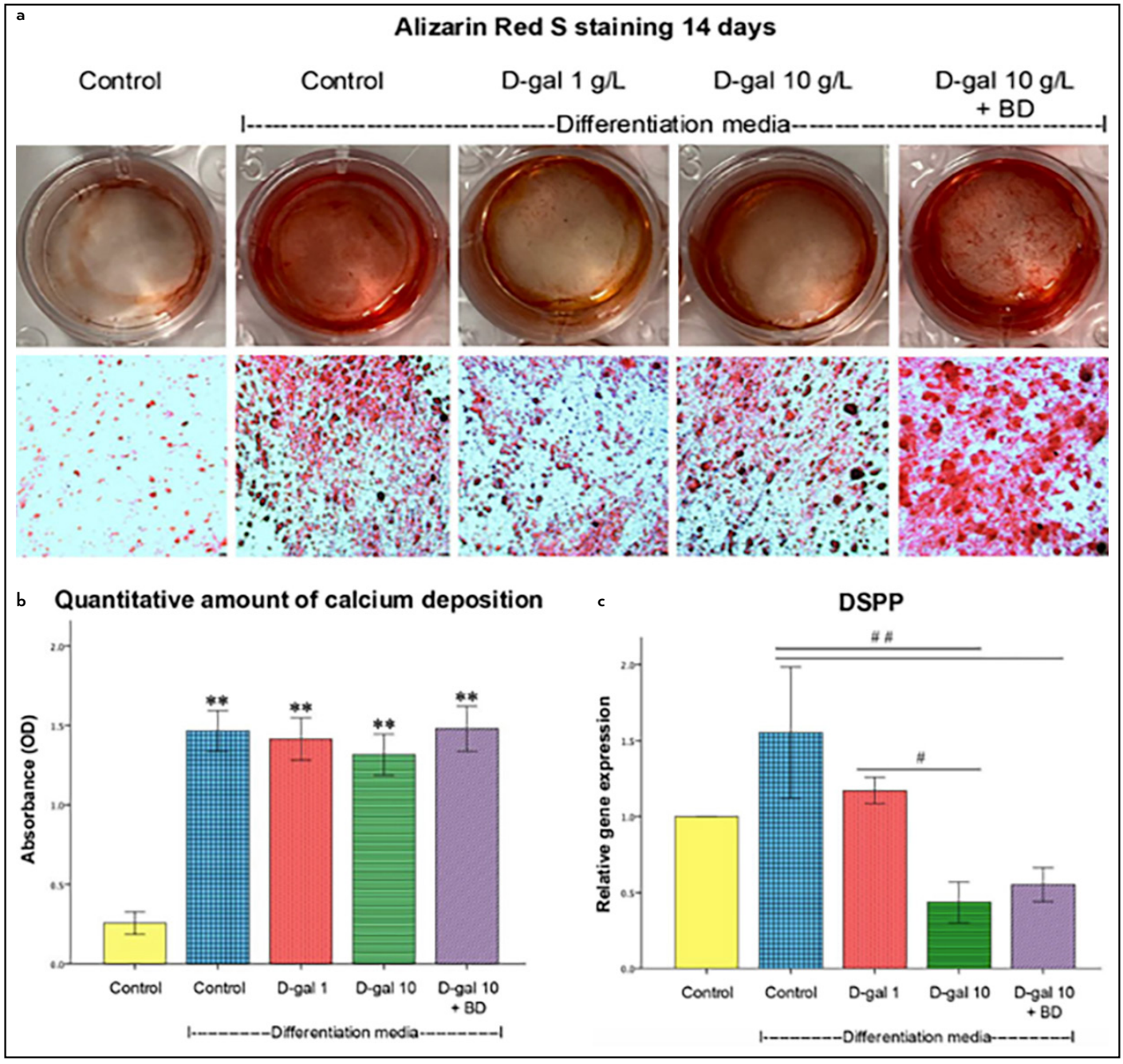

Figure 4. The effects of D-gal and co-incubation with BD on mineralization formation and odontogenic differentiation for 14 days. (a) Mineralization deposition stained with Alizarin Red S staining. (b) Quantitative amount of calcium deposition. (c) The expression of DSPP gene by qRT-PCR

**: p<0.001 compared to the control group, #: p<0.05 compared to the different concentration, # #: p<0.01 compared to the different concentration. D-gal: D-galactose, BD: Biodentine, OD: Optical density, DSPP: Dentine sialophosphoprotein, qRT-PCR: Quantitative reverse-transcription polymerase chain reaction

Odontogenic differentiation-related gene expression

The greatest level of DSPP gene expression was observed in the control group that experienced odontogenic differentiation using differentiation media (1.55±0.43-fold change); however, the difference was not significant compared to the control with no differentiation (p=0.09) (Fig. 4c). The presence of 1 g/L D-gal slightly decreased the DSPP levels (1.17±0.09-fold change) compared to the control group that underwent differentiation induction, although no significant difference was detected (p=0.23) (Fig. 4c). Interestingly, the 10 g/L D-gal significantly affected the level of DSPP, as shown by the lowest DSPP expression (0.72±0.17-fold change, p<0.05 vs. control with differentiation and 1 g/L D-gal groups) (Fig. 4c). The addition of BD to 10 g/L D-gal did not improve the expression levels of DSPP compared to the 10 g/L D-gal group (0.37±0.02-fold change, p=0.71) (Fig. 4c).

DISCUSSION

The present study showed that HDPCs treated with D-gal exhibited various aging phenotypes, including a significant reduction in cell proliferation, marked evidence of SA-β-gal positive cells, and increased expression of both the p16 and p21 genes. The supplementation of BD significantly reduced these markers of aging but did not strongly affect the miner-

alization potential or DSPP gene expression in the D-gal-treated cells. The methods used in this study, including SA-β-gal staining and the detection of p16 and p21 genes via qRT-PCR, are well-established and effectively capture markers of cellular senescence. Additionally, the mineralization assay, which includes both qualitative images of mineral production and quantitative gene expression results, successfully highlights the impact of D-gal on odontogenic differentiation. These established techniques provide a strong understanding of the aging process in HDPCs. However, incorporating additional markers related to oxidative stress or apoptosis could further enhance the comprehensiveness of the findings.

Dental pulp is a crucial component of the tooth, preserving its vitality by maintaining its function, health, and self-protective properties. However, the aging process, either due to increasing age in patients or external injury to the tooth, can impair dental pulp function. Therefore, it is crucial to examine the characteristics of dental pulp under aging conditions. Among various methods to simulate the aging environment *in vitro*, D-gal is an attractive substance used to induce aging due to its simplicity and effectiveness, as confirmed in several previous studies (18, 19). However, it has never been tested in a dental pulp model. This study aimed to evaluate whether D-gal could be used to simulate the aging model of the pulp as the findings would be beneficial for developing future experimental models for new treatment approaches.

Optimal substance concentration is crucial for inducing *in vitro* aging. Our preliminary results (supplementary Figure 1 revealed that 100 g/L D-gal induction resulted in severe and extensive mortality of HDPCs and may affect the availability of cells for further studies. Therefore, this study selected lower concentrations of D-gal to induce cellular senescence without severely disturbing cell viability. This study showed that concentrations of 1 and 10 g/L D-gal could trigger cellular senescence in which significant reductions in cell proliferation were observed. Previous studies using mesenchymal stem cells derived from rat bone marrow also reported similar findings in the reduction of cell proliferation after cells were treated with 10 g/L D-gal (20, 21). It should be noted that the reduction in cell proliferation is one of the aging phenotypes that has also been observed in long-term cell culture, known as replicative senescence (22, 23). Other studies in HDPCs also demonstrated the decline of cell proliferation when cells were cultured for a long period, 60 days (11) and 145 days (9).

For aging phenotypes, the detection of SA-β-gal activity is extensively used to confirm cellular senescence. β-galactosidase is a lysosomal enzyme found within normal cells, where an increase in cellular lysosomal activity is typically observed at the senescent stage (24–26). Moreover, the detection of p16 and p21 genes, which are associated with pathways involved in senescence, is also widely accepted (3, 27). In this present study, a significant increase in SA-β-gal positive cells was observed after treating cells with various concentrations of D-gal for 48 hours. Both p16 and p21 gene expressions were found to be upregulated at 48 hours in HDPCs induced by 10 g/L D-gal. A similar study using p-Cresol on dog dental pulp stem cells generated results which showed that cellular senescence was induced, as confirmed by the increase in SA-β-gal activity, p21, IL-1, IL-8, and p53 when used at 500 µmol/L for 72 hours (12). Although only short-term results (24–72 hours) on cellular senescence were available in our study, our findings are consistent with other studies that evaluated replicative cellular senescence of HDPCs in long-term cultures of 60 days (11) and 145 days (9). Comparable results from those studies included a decrease in cell proliferation, the accumulation of SA-β-gal positive cells, and the upregulation of aging-related genes such as p16 and p21, which are similar to our findings in the short-term culture. Interestingly, higher expressions of these aging phenotypes, including SA-β-gal and p16ink4a, were also exhibited in the dental pulp tissue isolated from aged patients (64 years; 62–66 years) compared to younger donors (10).

Regarding the mineralization potential, one of the defensive mechanisms of the pulp is to generate reparative dentine against the exogenous stimuli (28, 29). Therefore, it is essential for dental pulp cells to have the capability to differentiate into mineralization-producing cells. In general, the regenerative potential of dental pulp tissue has been claimed to become impaired with age (30, 31). In this present study, HDPCs treated with D-gal exhibited a slight declination of mineralization potential with the reductions of DSPP gene expressions. Similar findings were also reported when 20 mg/mL D-gal was applied to isolated human apical papilla cells (32). The use of other substances, for example, p-Cresol (12) and hydrogen peroxide (8, 11) also impacted the mineralization/differentiation effects of the cells. In addition, the long-term culture of HDPCs for 60 days (11) and 145 days (9) showed the decreased expression of odontogenic markers and mineralization nodule formation.

Therefore, based on the above-mentioned findings and their relations, D-gal is a good candidate for inducing cellular aging in HDPCs *in vitro* since it promotes the expression of various aging phenotypes. Compared to other models, 10 g/L D-gal induction for 48 hours is less time-consuming when compared to a model with long-term culture. However, the use of other substances, for example, p-Cresol and hydrogen peroxide is also promising, but both substances induce several consequences including inflammation in dental pulp cells as reported by the expression of inflammatory markers such as IL-6 and TNF-α (12, 33). Additional studies are still needed to investigate the effects of D-gal on dental pulp cells in other aspects such as oxidative stress, and inflammation induction.

To date, regenerative treatment approaches are crucial in dentistry. VPT is one such treatment that aims to regenerate damaged dental pulp, relying on the self-reparative potential of healthy dental pulp. In the past, this treatment strategy was typically limited to young patients. However, with advanced technologies and innovative materials, these treatments have been extended to older patients. BD, a tricalcium silicate-based cement, is one of the most prominent materials used in this field. Its composition, with superior properties such as shortened setting time and reduced potential for tooth discoloration, has made it popular in endodontics as an alternative to mineral trioxide aggregates (MTA) (34, 35). Previous stud-

ies have suggested that BD can promote cell proliferation and trigger mineralization production (36, 37). Additionally, BD is capable of diminishing pro-inflammatory and elevating anti-inflammatory cytokines (38, 39).

In the present study, the effect of BD on aged dental pulp cells was examined using a D-gal induction model. The findings from this study showed that the application of BD in the D-gal-treated HDPCs helped to reduce the expression of various aging phenotypes, including the expression of SA-β-gal, p16, and p21. BD tended to recover the level of mineralization production and the expression of the DSPP gene. However, BD seemed to have no positive effect on the proliferation of D-gal-treated cells. Our findings are inconsistent with a recent study that investigated the effect of 0.2 mg/mL BD extract on dental pulp cells isolated from donors aged 60 and above, reporting that BD extract promoted cell proliferation (40). Since the aged HDPCs in our study were derived from D-gal-induced aging, whereas the other study isolated cells from patients aged 60 and above, the cell models used are different. Additionally, the previous study did not report the effects of BD on cellular senescence markers. Therefore, further investigations are needed to confirm the effect of BD extract on the cell proliferation of aged HDPCs. To the best of our knowledge, there is still a lack of research exploring the effects of BD on cellular senescence. The results from our present study confirmed that BD could protect against the cellular senescence of dental pulp cells induced by D-gal, which suggests that BD has potential for use in the elderly. Future studies regarding the mechanism of BD on cellular senescence, for example, its potential for reactive oxygen species (ROS) reduction or other pathways, should be conducted.

CONCLUSION

Ten g/L of D-gal for 48 hours can effectively induce aging phenotypes in HDPCs, as evidenced by the reduction in cell proliferation, the increase in SA-β-gal positive cells, and the upregulation of p16 and p21 senescence-related genes. Co-incubation with BD extract reduced the expression of these aging phenotypes.

Disclosures

Acknowledgments: The authors would like to thank Adjunct Professor Richard L. Wilson, Faculty Consultant at the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand, for his assistance with the English language editing of this manuscript.

Ethics Committee Approval: The study was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University Ethics Committee (no: 83/2020, date: 25/11/2020).

Authorship Contributions: Concept – T.S., P.C.; Design – T.S., P.C.; Supervision – T.S., P.C.; Funding – P.C.; Materials – S.S., T.S., P.C.; Data collection and/or processing – S.S.; Data analysis and/or interpretation – S.S., S.V., T.S., P.C.; Literature search – S.S.; Writing – S.S., S.V., T.S., P.C.; Critical review – S.S., S.V., T.S., P.C.

Conflict of Interest: All authors declared no conflict of interest.

Use of AI for Writing Assistance: No AI technologies utilized.

Financial Disclosure: The research grant for this project was partially supported by the Thai Endodontic Association, the Fundamental Fund of Thailand Science Research and Innovation (TSRI), the CMU Mid-career Research Fellowship program, and the Faculty of Dentistry Research Funding, Chiang Mai University, Chiang Mai, Thailand.

Peer-review: Externally peer-reviewed.

REFERENCES

1. Murray PE, Stanley HR, Matthews JB, Sloan AJ, Smith AJ. Age-related odontometric changes of human teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002; 93(4):474–82. [CrossRef]
2. Amaya-Montoya M, Perez-Londono A, Guatibonza-Garcia V, Vargas-Villanueva A, Mendivil CO. Cellular senescence as a therapeutic target for age-related diseases: a review. Adv Ther 2020; 37(4):1407–24. [CrossRef]
3. Morsczeck C. Cellular senescence in dental pulp stem cells. Arch Oral Biol 2019;99:150–5. [CrossRef]
4. Duncan HF. Present status and future directions-vital pulp treatment and pulp preservation strategies. Int Endod J 2022; 55(Suppl 3):497–511. [CrossRef]
5. Taha NA, Abdelkhader SZ. Outcome of full pulpotomy using Biodentine in adult patients with symptoms indicative of irreversible pulpitis. Int Endod J 2018; 51(8):819–28. [CrossRef]
6. Wang K, Li L, Wu J, Qiu Q, Zhou F, Wu H. The different expression profiles of microRNAs in elderly and young human dental pulp and the role of miR-433 in human dental pulp cells. Mech Age Dev 2015; 146-148:1–11. [CrossRef]
7. Ma D, Ma Z, Zhang X, Wang W, Yang Z, Zhang M, et al. Effect of age and extrinsic microenvironment on the proliferation and osteogenic differentiation of rat dental pulp stem cells *in vitro*. J Endod 2009; 35(11):1546–53. [CrossRef]
8. Choi YJ, Lee JY, Chung CP, Park YJ. Cell-penetrating superoxide dismutase attenuates oxidative stress-induced senescence by regulating the p53-p21(Cip1) pathway and restores osteoblastic differentiation in human dental pulp stem cells. Int J Nanomedicine 2012; 7:5091–106. [CrossRef]
9. Nozu A, Hamano S, Tomokiyo A, Hasegawa D, Sugii H, Yoshida S, et al. Senescence and odontoblastic differentiation of dental pulp cells. J Cell Physiol 2018; 234(1):849–59. [CrossRef]
10. Iezzi I, Cerqueni G, Licini C, Lucarini G, Mattioli Belmonte M. Dental pulp stem cells senescence and regenerative potential relationship. J Cell Physiol 2019; 234(5):7186–97. [CrossRef]
11. Lee YH, Kim GE, Cho HJ, Yu MK, Bhattarai G, Lee NH, et al. Aging of *in vitro* pulp illustrates change of inflammation and dentinogenesis. J Endod 2013; 39(3):340–5. [CrossRef]
12. Zayed M, Iohara K. Effects of p-cresol on senescence, survival, inflammation, and odontoblast differentiation in canine dental pulp stem cells. Int J Mol Sci 2020; 21(18):6931. [CrossRef]
13. Acosta PB, Gross KC. Hidden sources of galactose in the environment. Eur J Pediatr 1995; 154(7 Suppl 2):S87–92. [CrossRef]
14. Ullah F, Ali T, Ullah N, Kim MO. Caffeine prevents d-galactose-induced cognitive deficits, oxidative stress, neuroinflammation and neurodegeneration in the adult rat brain. Neurochem Int 2015; 90:114–24. [CrossRef]
15. Azman KF, Zakaria R. D-Galactose-induced accelerated aging model: an overview. Biogerontology 2019; 20:763–82. [CrossRef]
16. Cebe T, Atukeren P, Yanar K, Kuruç AI, Ozan T, Kunbaz A, et al. Oxidation scrutiny in persuaded aging and chronological aging at systemic redox homeostasis level. Exp Gerontol 2014; 57:132–40. [CrossRef]
17. Weekate K, Chuenjitkuntaworn B, Chuveera P, Vaseenon S, Chompu-Inwai P, Ittichaicharoen J, et al. Alterations of mitochondrial dynamics, inflammation and mineralization potential of lipopolysaccharide-induced human dental pulp cells after exposure to N-acetyl cysteine, Biodentine or ProRoot MTA. Int Endod J 2021; 54(6):951–65. [CrossRef]
18. Shwe T, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Role of D-galactose-induced brain aging and its potential used for therapeutic interventions. Exp Gerontol 2018; 101:13–36. [CrossRef]
19. Bo-Htay C, Palee S, Apaijai N, Chattipakorn SC, Chattipakorn N. Effects of D-galactose-induced ageing on the heart and its potential interventions. J Cell Mol Med 2018; 22(3):1392–410. [CrossRef]
20. Zhang D, Yan B, Yu S, Zhang C, Wang B, Wang Y, et al. Coenzyme Q10 inhibits the aging of mesenchymal stem cells induced by D-galactose through Akt/mTOR signaling. Oxid Med Cell Longev 2015; 2015:867293. [CrossRef]
21. Yang M, Teng S, Ma C, Yu Y, Wang P, Yi C. Ascorbic acid inhibits senescence in mesenchymal stem cells through ROS and AKT/mTOR signaling. Cytotechnology 2018; 70(5):1301–13. [CrossRef]
22. Mehrazarin S, Oh JE, Chung CL, Chen W, Kim RH, Shi S, et al. Impaired odontogenic differentiation of senescent dental mesenchymal stem cells is associated with loss of Bmi-1 expression. J Endod 2011; 37(5):662–6. [CrossRef]

23. Li L, Zhu YQ, Jiang L, Peng W. Increased autophagic activity in senescent human dental pulp cells. Int Endod J 2012; 45(12):1074–9. [CrossRef]
24. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell 2013; 153(6):1194–217. [CrossRef]
25. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of cellular senescence. Trends Cell Biol 2018; 28(6):436–53. [CrossRef]
26. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, et al. Cellular senescence: defining a path forward. Cell 2019; 179(4):813–27. [CrossRef]
27. Khan SS, Singer BD, Vaughan DE. Molecular and physiological manifestations and measurement of aging in humans. Aging Cell 2017; 16(4):624–33. [CrossRef]
28. Hao J, Ramachandran A, George A. Temporal and spatial localization of the dentin matrix proteins during dentin biomineralization. J Histochem Cytochem 2009; 57(3):227–37. [CrossRef]
29. Iohara K, Zayed M, Takei Y, Watanabe H, Nakashima M. Treatment of pulpectomized teeth with trypsin prior to transplantation of mobilized dental pulp stem cells enhances pulp regeneration in aged dogs. Front Bioeng Biotechnol 2020; 8:983. [CrossRef]
30. Shiba H, Nakanishi K, Rashid F, Mizuno N, Hino T, Ogawa T, et al. Proliferative ability and alkaline phosphatase activity with *in vivo* cellular aging in human pulp cells. J Endod 2003; 29(1):9–11. [CrossRef]
31. Couve E, Osorio R, Schmachtenberg O. Mitochondrial autophagy and lipofuscin accumulation in aging odontoblasts. J Dent Res 2012; 91(7):696–701. [CrossRef]
32. Teawcharoensopa C, Srisuwan T. The potential use of ascorbic acid to recover the cellular senescence of lipopolysaccharide-induced human apical papilla cells: an *in vitro* study. Clin Oral Investig 2023; 28(1):49. [CrossRef]
33. Vaseenon S, Weekate K, Srisuwan T, Chattipakorn N, Chattipakorn S. Observation of inflammation, oxidative stress, mitochondrial dynamics, and apoptosis in dental pulp following a diagnosis of irreversible pulpitis. Eur Endod J 2023; 8(2):148–55. [CrossRef]
34. Koubi G, Colon P, Franquin JC, Hartmann A, Richard G, Faure MO, et al. Clinical evaluation of the performance and safety of a new dentine substitute, Biodentine, in the restoration of posterior teeth - a prospective study. Clin Oral Investig 2013; 17(1):243–9. [CrossRef]
35. Camilleri J. Investigation of Biodentine as dentine replacement material. J Dent 2013; 41(7):600–10. [CrossRef]
36. Laurent P, Camps J, About I. Biodentine(TM) induces TGF-beta1 release from human pulp cells and early dental pulp mineralization. Int Endod J 2012; 45(5):439–48. [CrossRef]
37. Youssef AR, Emara R, Taher MM, Al-Allaf FA, Almalki M, Almasri MA, et al. Effects of mineral trioxide aggregate, calcium hydroxide, biodentine and Emdogain on osteogenesis, Odontogenesis, angiogenesis and cell viability of dental pulp stem cells. BMC Oral Health 2019; 19(1):133. [CrossRef]
38. Giraud T, Jeanneau C, Rombouts C, Bakhtiar H, Laurent P, About I. Pulp capping materials modulate the balance between inflammation and regeneration. Dent Mater 2019; 35(1):24–35. [CrossRef]
39. Erakovic M, Duka M, Bekic M, Tomic S, Ismaili B, Vucevic D, et al. Anti-inflammatory and immunomodulatory effects of Biodentine on human periapical lesion cells in culture. Int Endod J 2020; 53(10):1398–412. [CrossRef]
40. Wang MC, Chang KW, Lin SC, Hung PS. Biodentine but not MTA induce DSPP expression of dental pulp cells with different severity of LPS-induced inflammation. Clin Oral Investig 2023; 27(3):1207–14. [CrossRef]