



Influence of Hypoxic Condition on Cytotoxicity, Cellular Migration, and Osteogenic Differentiation Potential of Aged Periodontal Ligament Cells

Sukrit Jaiklaew¹ Kallapat Tansriratanawong¹ 

¹Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Eur J Dent

Address for correspondence Kallapat Tansriratanawong, DDS, MSc, PhD, Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University, No. 6, Yothi Road, Ratchathewi District, Bangkok 10400, Thailand (e-mail: kallapat.tan@mahidol.ac.th).

Abstract

Objective This study aimed to investigate and compare the influence of hypoxic conditions on cytotoxicity, cellular migration, and osteogenic differentiation of aged periodontal ligament (PDL) cells.

Materials and Methods Isolated human PDL cells from aged and young subjects were cultured under hypoxic conditions, which were treated with hydrogen peroxide (H₂O₂) (0, 25, 50, 100, 200, and 500 μM). To assess cytotoxicity, lactate dehydrogenase release was determined by the optical density at 490 nm, and the percentage of cell death was calculated. An *in vitro* wound healing assay was performed over 24 to 48 hours for cellular migration determination. Osteogenic differentiation was determined by alizarin red staining and osteogenic gene expression, including the expression of runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteopontin (OPN).

Results There was a significant difference in the percentage of cell death with high hypoxic condition (200 and 500 μM) compared to low hypoxic conditions on both day 1 and 2. The highest cellular migration was depicted at 50 μM in both young and aged groups of the *in vitro* wound healing assay. Osteogenic gene expression of RUNX2 in the aged group was increased at 25 and 50 μM hypoxic condition at day 7, but the expression was gradually decreased after 14 days. On the contrary, the expression of ALP and OPN in the aged group was increased at day 14. Only OPN had been found to be statistically significantly different when compared with gene expression at day 7 and 14 ($p < 0.05$). The results showed no statistically significant differences when compared with the young and aged groups in all genes and all concentrations.

Conclusion The concentration of low hypoxic condition (25–50 μM) was proposed to promote cell viability, cellular migration, and osteogenic differentiation in aged PDL cells. We suggested that the potential of aged PDL cells for use in cell therapy for periodontal regeneration might possibly be similar to that of young PDL cells.

Keywords

- ▶ periodontal ligament
- ▶ cell hypoxia
- ▶ cell migration assays
- ▶ osteogenesis
- ▶ cell differentiation

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Introduction

Periodontal disease is a chronic inflammatory disease in which bacteria infect, invade, and destroy the periodontium, including the gingiva, cementum, periodontal ligament (PDL), and alveolar bone. It is initiated by periodontal pathogenic bacterial stimulation from dental biofilms that deposit on the surface of teeth, implants, or prostheses and result in an imbalance of the environment within the biofilms, and eventually cause the loss of alveolar bone support. Disease progression is not only dependent on the pathogenic bacteria but also modified by host susceptibility and the immune response.^{1,2} The immune response can be altered and modified by several factors, such as genetics, environment, behavior, and aging.² Various biological and physiological changes in immune responses have been suggested to be age-related, including increased serum C-reactive protein levels, changes in inflammatory mediators, and reduced levels of serum folate, which is a protective factor against chronic inflammatory diseases.³⁻⁵

Aging is the biological process of growing older and has been suggested to involve the accumulation of cellular damage over time. During the aging process, the periodontium changes physically, biologically, and functionally, which leads to enhanced bacterial invasion, and the progression of disease may be faster with increasing age, a low rate of cell turnover, susceptibility to inflammation, and impaired dexterity that leads to poor biofilm control.^{5,6} Thus, age has been suggested to be a risk determinant for periodontal disease, with an odds ratio of 1.05 to 2.29.^{7,8} Nevertheless, it has been established that these age-related factors of periodontal disease have negligible effects on an individual's responsiveness to periodontal treatment if the patient has good biofilms control.⁹ Compared with younger patients, aged patients with periodontitis showed no difference in the clinical response to nonsurgical treatment.¹⁰ Regarding cell-based therapy for periodontal regeneration, the differentiation potential of osteoblastic progenitor cells derived from aged individuals is highly variable, and it remains unclear whether aged patients have worse regenerative results or are similar to those of young patients.¹¹⁻¹³

One possible mechanism drawn from the mitochondrial theory of aging is because mitochondria can induce oxidative damage, it can result in reactive oxygen species (ROS) production. Overabundant ROS has harmful effects on mitochondrial function and causes cumulative damage; therefore, cells try to compensate by producing antioxidants. If the damage is overwhelming, the mitochondria are not able to supply enough energy and generate oxygen-deprived conditions, which contribute to the aging process and lead to hypoxia.¹⁴ However, low concentrations of exogenous H₂O₂ and low levels of ROS contribute to hypoxic conditions in mesenchymal stem cells (MSCs) and have been reported to promote MSC proliferation and migration via the extracellular-signal-regulated kinase 1/2 and Jun-1/2 pathways.¹⁵⁻¹⁸ Additionally, growth factors such as platelet-derived growth factor-BB and vascular endothelial growth factor (VEGF) were suggested to be inducible and to exhibit enhanced

expression under low-level ROS conditions, which also promotes the proliferation and migration of MSCs.¹⁹⁻²¹ The VEGF gene is highly expressed and enhances angiogenesis under hypoxic conditions via hypoxia-inducible factor-1 alpha (HIF-1 α) in PDL fibroblasts.^{22,23} Therefore, HIF was suggested to be a linking factor that may play a key role in inducing the overexpression of angiogenesis-related factors and contribute to osteogenic differentiation in PDL cells.²⁴

Evidence on low-level hypoxic conditions and their effects in PDL cells derived from aged subjects is limited. PDL cells were selected as candidate cells due to their progenitor or stem cell-like properties, including self-renewal, clonogenicity, multilineage differentiation, ease of harvest, and they may be a useful cell source for periodontal regeneration.²⁵ Moreover, although the prevalence of periodontal disease is increased in aging subjects due to the accumulation of damage, disease progression, and impaired periodontal wound healing in aged cells, the disease does not always progress. We structured the null hypothesis stating that the influence of hypoxic condition on cytotoxicity, cellular proliferation, cellular migration, and osteogenic differentiation of aged PDL cells does not differ from that of young PDL cells. Conversely, the alternative hypothesis is that a low level of hypoxia may promote periodontal regeneration in aged patients. Therefore, this study aimed to investigate and compare the influence of hypoxic conditions on cytotoxicity, cellular migration, and osteogenic differentiation of aged PDL cells.

Materials and Methods

Study Populations

All subjects who were enrolled in this study were asked to provide informed consent. The inclusion criteria for the aged group were subjects who were 60 years old or older. For comparison, the young group included subjects who were less than 30 years old. All subjects were clinically healthy or had well-controlled systemic diseases. The exclusion criteria were subjects who had uncontrolled systemic diseases that caused impaired healing and women who were pregnant or lactating. All studies were conducted according to the guidelines of the research ethics committee of Mahidol University, Bangkok, Thailand (COE.No. MU-DT/PY-IRB 2021/024.3007).

Human PDL Cell Isolation and Culture

Intact periodontal teeth, which demonstrated absence of bleeding on probing (probing depth \leq 4 mm) and had non-carious lesions on the root surface, were extracted from 10 subjects in each aged and young groups for orthodontic reasons, prosthodontic reasons, endodontic reasons, removal of wisdom teeth, or simple extraction at the Oral and Maxillofacial Surgery Clinic, Mahidol University, Bangkok, Thailand. The teeth were randomly used for at least three samples in each group in all experiments. The teeth were washed twice with phosphate-buffered saline (PBS), and PDL tissues were scraped and isolated from the middle one-third of the noncarious root surfaces with a sterile scalpel. The tissues were minced into small pieces and further isolated

within 24 hours by the outgrowth method. The isolated PDL cells were placed into 60-mm dishes (Corning, New York, United States) with 3 mL of culture medium that was prepared from Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, 0.05% glutamine (Gibco BRL, Carlsbad, California, United States), 0.5% penicillin/streptomycin (Gibco BRL), and 0.1% amphotericin B (Gibco BRL) as growth medium (GM) and cultured at 37°C in a 20% O₂ and 5% CO₂ atmosphere.

Fibroblast-like cells, which were derived from PDL tissues and migrated from the tissue fragments, were isolated and defined as primary PDL cells. Colony formation of PDL cells was investigated to characterize progenitor potential. After the PDL cells reached approximately 80 to 90% confluence, the cells were subcultured at a dilution of 1:3 and were defined as the 1st passage. The human PDL cell line was purchased, cultured according to the manufacturer's instructions, and used as a control (HPDLF, Catalog Number: 2630, ScienCell, California, United States). The 3rd to 7th passages of aged and young PDL cells were used in all experiments.

Senescence Assay

Senescence detection was performed with a senescent cell histochemical staining kit (Sigma-Aldrich, Missouri, United States) to evaluate and compare the primary senescence characteristics of the aged and young groups at the 3rd passage. The staining mixture was prepared by mixing 1 mL of staining solution, 125 µL of reagent B, 125 µL of reagent C, 0.25 mL of X-gal solution, and 8.5 mL of water. The cells from each group were seeded at a density of 5 × 10⁴ cells/1 mL/well in a 24-well plate with normal GM for 24 hours. Then, the GM was removed and the cells were washed twice with PBS. The fixation buffer was added to each well and incubated for 7 minutes at room temperature (25°C). Then, the cells were washed three times with PBS and a staining mixture was added to each well. The plate was incubated at 37°C without CO₂ for 24 hours (Thermo Fisher Scientific Inc., United States). The image of each well at 10× magnification under microscope was captured. Each picture was named. The name of each picture was randomly chosen for 10 images for each group and the blue-stained cells (senescent cells) were counted and calculated from each group with the following formula:

$$\text{Senescent cells (\%)} = \frac{\text{total number of stained cells}}{\text{total number of counted cells}} \times 100$$

Proliferation Assay

Cellular proliferation was examined by 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT; PanReac Appli Chem, Germany) for proliferation assay among control, aged, and young group. The 5 × 10³ cells suspension/well were seeded in 96-well plates (NUNC) and incubated for 24 hours. Supernatant was replaced by the fresh GM and cells from three groups were incubated under the same condition at 24 to 48 hours. Each well was added with 20 µL of prewarmed (37°C) MTT solution and cultured for 2 hours. Dimethyl sulfoxide was used for inhibiting the reaction by adding to each well at 37°C for 30 minutes. The optical

density (OD) of solution was measured at 570 nm of wavelength by enzyme-linked immunosorbent assay (ELISA) plate reader (Epoch, BioTek Instruments, Inc., United States).

Preparation of H₂O₂ for Hypoxic Cell Culture Conditions

The stock solution was prepared from a 10-mM H₂O₂ solution in sterile distilled water. Then, the stock solution was diluted in GM until it reached the final concentration, which was 25, 50, 100, 200, or 500 µM for all experiments. For the hypoxic condition, PDL cells were seeded in 24-well plates at an initial density of 2 × 10⁵ cells/mL for 24 hours, and GM with 25, 50, 100, 200, and 500 µM H₂O₂ was added to each well. In contrast, the normoxic condition was defined as PDL cells seeded in GM at the same cell density in the absence of H₂O₂. PDL cells from all conditions were cultured at 37°C in a 20% O₂ and 5% CO₂ atmosphere.

Cytotoxicity Assay

Cytotoxicity was measured by the release of the enzyme lactate dehydrogenase (LDH) in the GM (Roche, Mannheim, Germany). LDH is a stable cytoplasmic enzyme that is found in all cells and is rapidly released into the cell culture supernatant when the plasma membrane is damaged. PDL cells were seeded in 24-well plates at an initial density of 2 × 10⁵ cells/mL for 24 hours, and then the supernatant was pooled and collected into a 15-mL centrifuge tube (Corning, New York, United States) for each group. The 50-µL of supernatant from each group at all concentrations was transferred to a 96-well plate in triplicate. The normoxic GM was used as a negative control, and the wells of the PDL cell line, which were supplemented with 5 µL of lysis buffer (2% Triton X-100 solution) and incubated for 15 minutes, were used as a positive control. Then, 50 µL of the LDH assay reagent was added to the supernatants as a substrate. The reagents were mixed by gentle shaking for 30 seconds. The plate was incubated at 37°C in a 20% O₂ and 5% CO₂ atmosphere for 10 minutes in the incubator (Thermo Fisher Scientific Inc.). Then, 100 µL of stop solution (hydrochloric acid 1 N) was added to each well, and the reagent was mixed by shaking for 30 seconds. The absorbance of each well was measured at 490 nm (setting the background absorbance at 690 nm) within 1 hour. Cytotoxicity, which was induced by H₂O₂, was observed. The percentage of cell death (% cytotoxicity) was calculated from the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{OD(490nm)experiment} - \text{OD(490nm)low control}}{\text{OD(490nm)high control} - \text{OD(490nm)low control}} \times 100$$

Cellular Migration (*In Vitro* Wound Healing Assay)

The *in vitro* wound healing assay was performed in triplicate. PDL cells in each group were seeded in 24-well plates at a density of 2 × 10⁵ cells/well and cultured as a monolayer for 1 to 2 days until the cells reached 90 to 100% confluence. PDL cells were scratched vertically with a 1,000-µL pipette tip to create an artificial wound. For the baseline area (day 0), the medium was removed immediately after scratching. The cells were washed twice with PBS and fixed with 100% methanol for 10 minutes. After fixation, the PDL cells were washed

twice with PBS to remove residual methanol, stained with 10% Giemsa (Merck, Missouri, United States) for 10 minutes and finally rinsed with water. The area of the artificial wound was assessed under an inverted microscope (Olympus CKX53, Shinjuku, Japan) using 4× magnification. The artificially wounded PDL cell cultures were subjected to normoxic and hypoxic conditions for 24 and 48 hours, respectively. Afterward, the artificially wounded cells were washed twice with PBS, fixed with 100% for 10 minutes, washed twice with PBS, and stained with 10% Giemsa. The areas of day 0, day 1, and day 2 groups were measured, and the surface area was estimated with ImageJ (National Institutes of Health, United States) and calculated with the following formula:

$$\text{Percentage of wound closure (\%)} = \frac{\text{total area of cells in the gaps}}{\text{total area of the gaps}} \times 100$$

Osteogenic Differentiation

PDL cells were seeded at a density of 2×10^5 cells/well in 6-well plates with normal GM until they reached 80 to 90% confluence. Then, PDL cell cultures were subjected to normoxic and hypoxic osteogenic induction media, which included 100 nM dexamethasone (Sigma–Aldrich), 50 μ M ascorbic acid (Sigma–Aldrich), and 10 mM β -glycerophosphate (Sigma–Aldrich) in GM. Osteogenic differentiation of the aged and young groups was detected by mineralization assays and gene expression at 7 and 14 days.

Alizarin Red Staining

Alizarin red staining (ARS) solution was prepared by dissolving 3 g alizarin red (Sigma–Aldrich) in 100 mL distilled water and gently mixing. The pH was adjusted with hydrochloric acid (RCI Labscan, Ireland) until it was approximately 4.1 to 4.3. PDL cells with osteogenic induction were washed twice with PBS (VWR, France) and fixed with 10% buffer formalin (Loba Chemie, India) for 10 minutes. ARS solution was added to each well and incubated at room temperature for 45 minutes protected from light. After the ARS solution was aspirated, the cells were washed with distilled water several times and inspected with a phase contrast microscope. For the quantification assay, 800 μ L of 10% acetic acid (RCI Labscan) was added to osteogenic induction in each well and incubated at room temperature for 30 minutes. Then, the cells were transferred to a 1.5-mL microcentrifuge tube and vortexed for 30 seconds (Vortex2, Scientific Industries, New York, United States). The solution with cells was heated at 85°C for 10 minutes, incubated on ice for 5 minutes, and centrifuged at $20,000 \times g$ for 15 minutes (Hettich Universal, Singapore). Then, 500 μ L of the supernatant was transferred to a new tube. Next, 200 μ L of 10% ammonium hydroxide (Loba Chemie) was added to neutralize the acid. A 150- μ L aliquot of the sample was transferred into a 96-well plate, and the absorbance was read at 405 nm in ELISA plate reader (Epoch, BioTek Instruments, Inc.). The assay was performed for 7 and 14 days. The ARS standard solution was prepared from 100 μ L of 40 mM ARS solution and 900 μ L of distilled water and adjusted pH until 4.1 to 4.3 with 10% ammonium hydroxide and 10% acetic acid to make 1 mL of 4 mM ARS. The solution was mixed well in 1.5 mL microcentrifuge tubes.

Then, 500 μ L of standard solution was added to each tube; 500 μ L of 4 mM ARS solution was added to obtain 2 mM ARS standard in the first tube and 500 μ L of 2 mM ARS solution was transferred to the next tube to obtain 1 mM ARS solution. The procedure was repeated, and the solution serially diluted to 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.0313 mM and blank. A total of 150 μ L of each concentration was transferred to a 96-well plate in triplicate. The absorbance at 405 nm was read. These absorbance values were plotted as points to illustrate the relationship between concentration and absorbance. A quantification standard curve was generated from these points and the resulting equation was utilized for quantification purposes.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

Osteogenic markers in differentiated cells were evaluated with real-time reverse transcriptase polymerase chain reaction (RT-PCR) to compare gene expression between day 7 and day 14 for aged and young groups. Osteogenic induction and noninduction underwent osteogenic differentiation under normoxic and hypoxic conditions. The markers were HIF-1 α , runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteopontin (OPN), and the primer sequences are presented in **–Supplementary Table S1** (available in the online version). The levels of β -actin expression were used as an endogenous control. The ribonucleic acid (RNA) from the samples was prepared with FavorPrep (Favogen Biotech Corp., Ping-Tung, Taiwan) and determined from the absorbance at 260/280 nm (NanoDrop, Thermo Fisher Scientific, Massachusetts, United States). Then, 500 ng of RNA was used along with forward and reverse PCR primers to amplify HIF-1 α and osteogenesis-related genes (CFX96 touch real-time PCR detection system, Bio-Rad, California, United States). Real-time RT-PCR was performed with qPCRBIO SyGreen 1-step Go Lo-ROX (PCR Biosystems, London, United Kingdom). Reactions were done with $2 \times$ qPCRBIO SyGreen 1-step Mix, $20 \times$ RTase Go, primers, and distilled water. Reverse transcription was performed for 10 minutes at 45°C. The polymerase activation was activated at 95°C for 2 minutes. The amplification consisted of 50 cycles of 95°C for 5 seconds, annealing at 56 to 60°C for 30 seconds, and a final extension step at 95°C for 15 seconds. The assay was performed in duplicate and data were derived from quantitative data and compared with endogenous genes. The result of real-time RT-PCR was shown as increasing fluorescence signals and was measured as the Ct value. The relative fold change in gene expression was calculated with the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

All data were analyzed for a normal distribution with the Shapiro–Wilk test. Cell cytotoxicity, cellular migration assay, quantification of ARS, and real-time RT-PCR were presented as the median, 25th percentile, and 75th percentile. The senescence data was illustrated as the mean \pm standard deviation. Kruskal–Wallis test was used to compare the percentage of cell death and cellular migration assay among groups. Cellular proliferation was analyzed by one-way analysis of variance for

comparing means among control, young, and aged group. The Mann–Whitney *U* test was used to compare the concentration of ARS and relative gene expression between two groups. Wilcoxon signed rank test was used to compare the concentration of ARS and relative gene expression between 7 and 14 days. Friedman test was used to compare the percentage of cell death, percentage cellular migration, concentration of ARS, and relative gene expression among concentrations. All possible pairwise comparisons were performed using Bonferroni adjustment. The cytotoxicity assay, cellular migration assay, and quantification of ARS were performed in triplicate, while real-time RT-PCR was performed in duplicate. SPSS program ver. 28 (SPSS Inc., Chicago, Illinois, United States) was used for the analyses. A *p*-value of < 0.05 was considered statistically significant.

Results

Senescent Characteristics of PDL Cells

Senescent staining was performed for the PDL cell line (control), the young, and aged groups (►Fig. 1A–C), respectively (magnification 4 ×). The higher magnification (10 ×) of the aged group is depicted in ►Fig. 1D. Beta-galactosidase is a marker of senescent cells and displayed in blue in the nucleus (►Fig. 1C, D). The results showed that cellular senescence was exclusively observed in the aged group, while senescence was not observed in the control and young groups. The percentages of stained cells in the control, young, and aged groups were 0, 0, and 1.72%, respectively (►Fig 1E).

Cellular Proliferation of PDL Cells

PDL cells of young and aged groups demonstrated lower proliferation potential when compared to the control group

at 24 hours. There were no statistically significant differences among the three group at 24 hours (*p* > 0.05, ►Fig. 1F). By time-dependence at 48 hours, the young group showed the highest proliferation rate when compared to the control and aged group, but there were no statistically significant differences among the three groups (*p* > 0.05, ►Fig. 1F).

Increasing Amounts of Cell Death at High Concentrations

Cytotoxicity was demonstrated by the percentage of cell death in the LDH assay at 24 hours (day 1) and 48 hours (day 2). Cytotoxicity from 25 to 500 μM of all groups was trended to gradual increase by H₂O₂ concentration dependence. Statistically significant differences were demonstrated in the percentage of cell death with high concentrations (200 and 500 μM) compared to low concentration on day 1 and 2 (►Fig. 2A, B, *p* < 0.05).

Similar Wound Closure in the Aged and Young Groups Under Low-Level Hypoxic Conditions

An *in vitro* wound healing assay was performed to analyze the cellular migration of PDL cells after treatment with H₂O₂ in the aged and young groups. The aged and young groups exhibited similar cellular migration rates of all concentrations on day 1 and 2 (►Fig. 3A, B). The trend of cellular migration was decreasing when higher concentrations were used in all groups. Interestingly, the control group demonstrated strong cellular migration even when H₂O₂ was applied, and the percentages varied from 54.78 to 78.55% on day 2, with a significant difference compared to either the aged or young groups at all concentrations (*p* < 0.05 ►Fig. 3A–C). However, there was no statistically significant difference between aged and young groups.

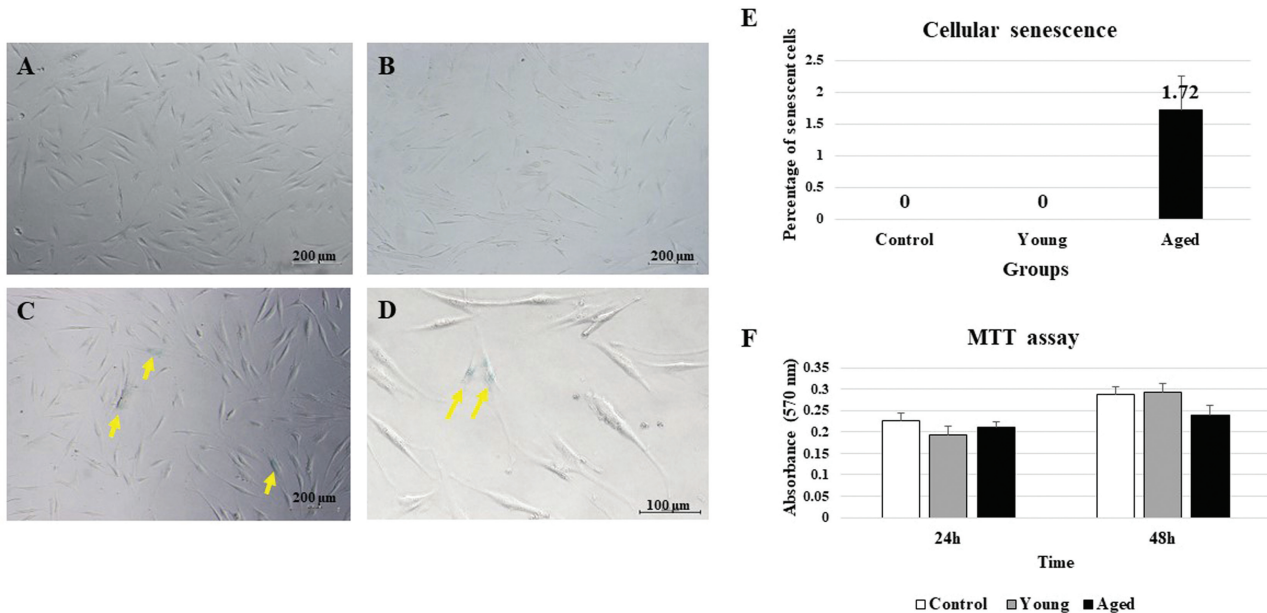


Fig. 1 Senescent assay. (A) Image from phase contrast microscope of control (periodontal ligament [PDL] cell line), (B) young group, and (C) aged group. (A–C) 4× magnification and scale bar = 200 μm. (D) Aged group with 10× magnification. Scale bar = 100 μm. (E) Percentage of senescent cells in passage 3. (F) Cellular proliferation of control, young, and aged groups (*n* = 10).

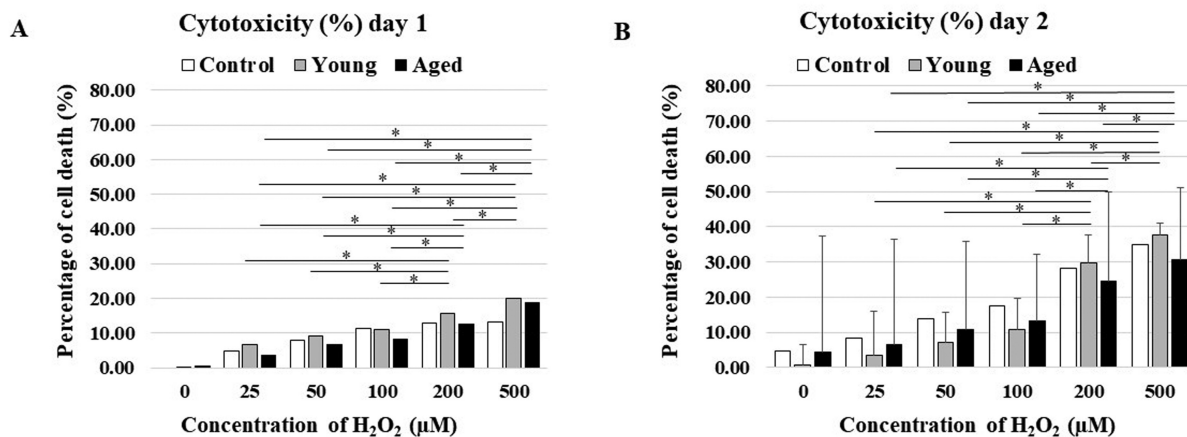


Fig. 2 Comparing each concentration of cytotoxic assay. (A) Chart showed percentage of cytotoxicity on day 1. (B) Chart showed percentage of cell death on day 2. The differences in each group were significant as shown by star (*). Concentration of 200 and 500 μM were significantly different from other concentrations ($p < 0.05$) for both of day 1 and day 2 ($n = 3$).

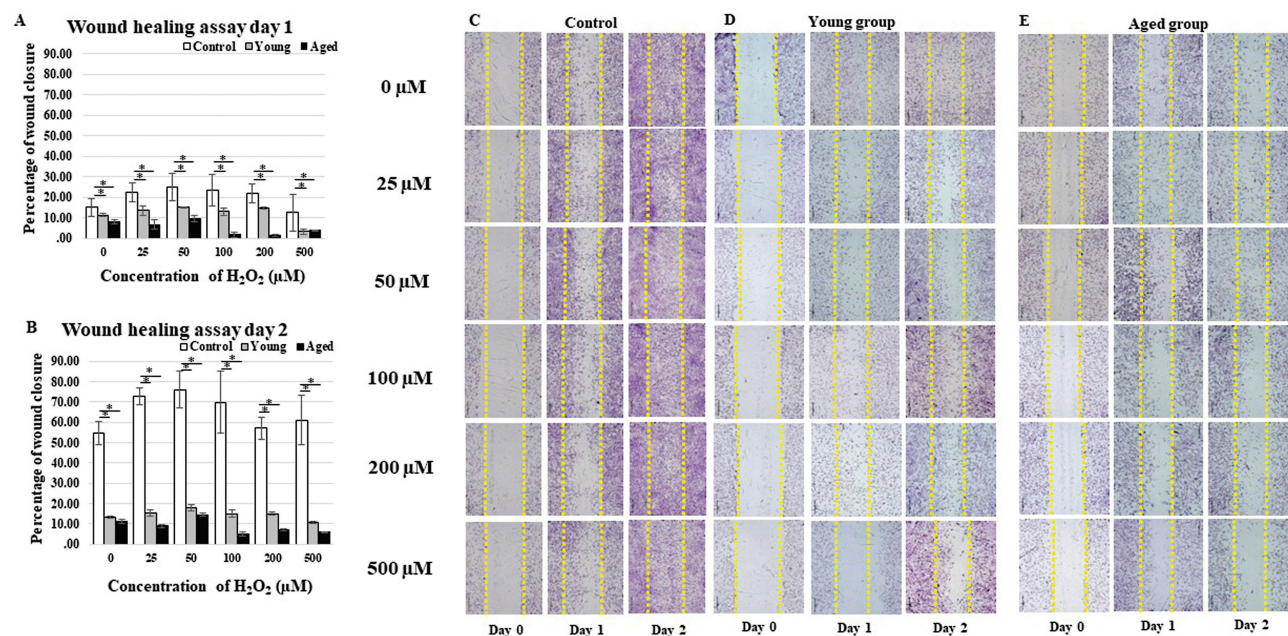


Fig. 3 For cellular migration in each concentration (0, 25, 50, 100, 200, and 500 μM) and day: (A) day 1 and (B) day 2. Control had a significant difference compared to the aged and young groups at all concentrations on day 1 and 2 ($p < 0.05$). The differences were significant as shown by star (*). Pictures depicted *in vitro* wound healing staining with Giemsa (C) control, (D) young, and (E) aged group (scale bar = 200 μm, $n = 10$).

Mineralization Determined by Alizarin Red Staining

The standard curve of ARS is plotted according to the equation $ARS = 0.751 (OD\ 405\ nm) + 0.0589$. Quantification and staining of ARS were performed as shown in ▶Fig. 4A–C. On day 7, 50 μM in the aged and young groups significantly differed from 500 μM in the aged and young groups ($p = 0.016$ and 0.016 , respectively, ▶Fig. 4A). None of the concentrations of ARS were significantly different between the aged and young groups at day 14 ($p \geq 0.05$, ▶Fig. 4B). Although there were no statistically significant difference at all concentrations, interestingly, day 14 had higher mineralization in the aged group than in the young group (▶Fig. 4B).

Gene Expression of HIF-1α and Osteogenic Genes

Gene expression of the HIF-1α gene is displayed in ▶Fig. 5A, B. Interestingly, the highest expression was observed at a concentration of 50 μM in the aged group on day 7 and at a concentration of 25 μM in the aged group on day 14, respectively (▶Fig. 5A, B), and in the young group at the same concentrations (50 μM) on day 14 (▶Fig. 5B), but they were no statistically significant differences when compared to other groups and concentrations.

When the gene expression of osteogenic genes was examined (▶Fig. 5C–H). The RUNX2 gene showed the highest expression at a concentration of 50 μM on day 7 and at a concentration of 25 μM on day 14, and there was no

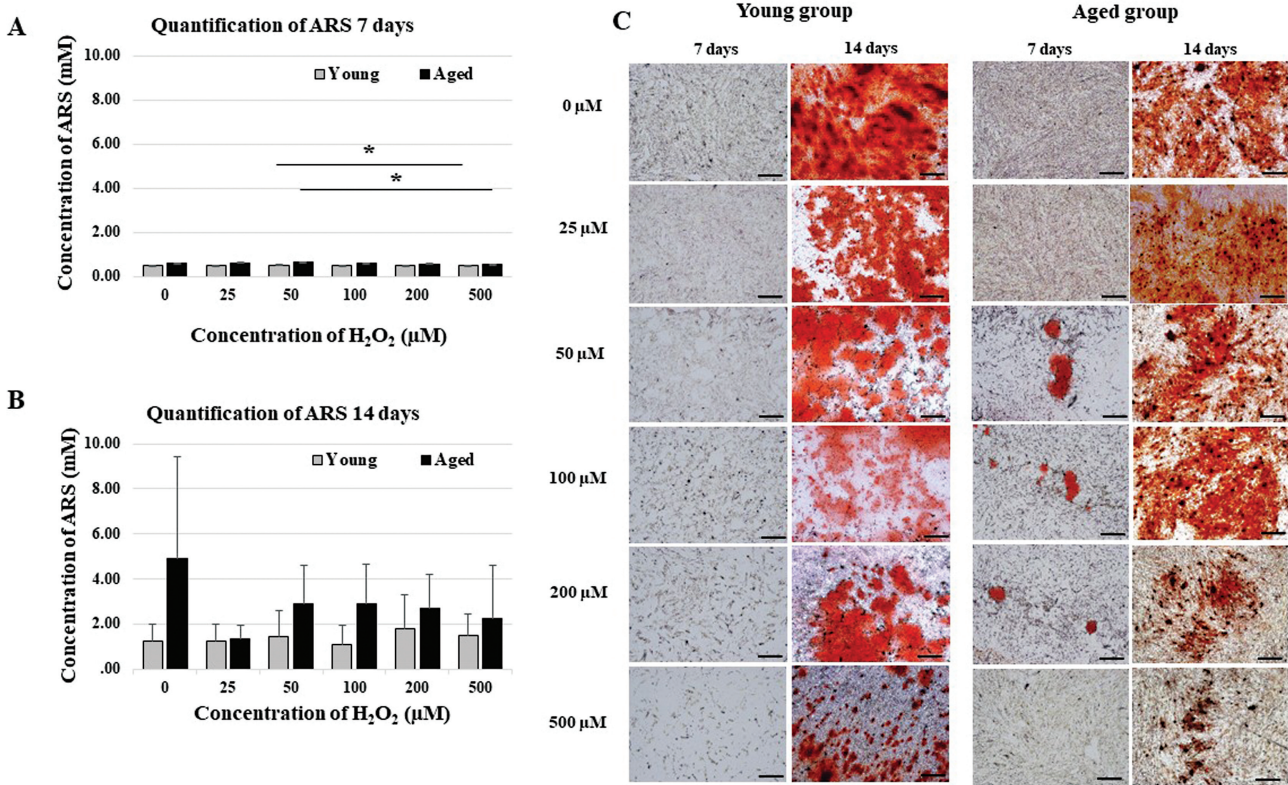


Fig. 4 Quantification of alizarin red staining (ARS). (A) At 7 days, the concentration of ARS (mM) for 50 μM concentration of both aged and young groups were significantly different when compared to 500 μM ($p = 0.016$ and 0.016 , respectively). (B) At 14 days, the concentration of ARS for all concentrations were not significant ($p \geq 0.05$, scale bar = 200 μm, $n = 3$).

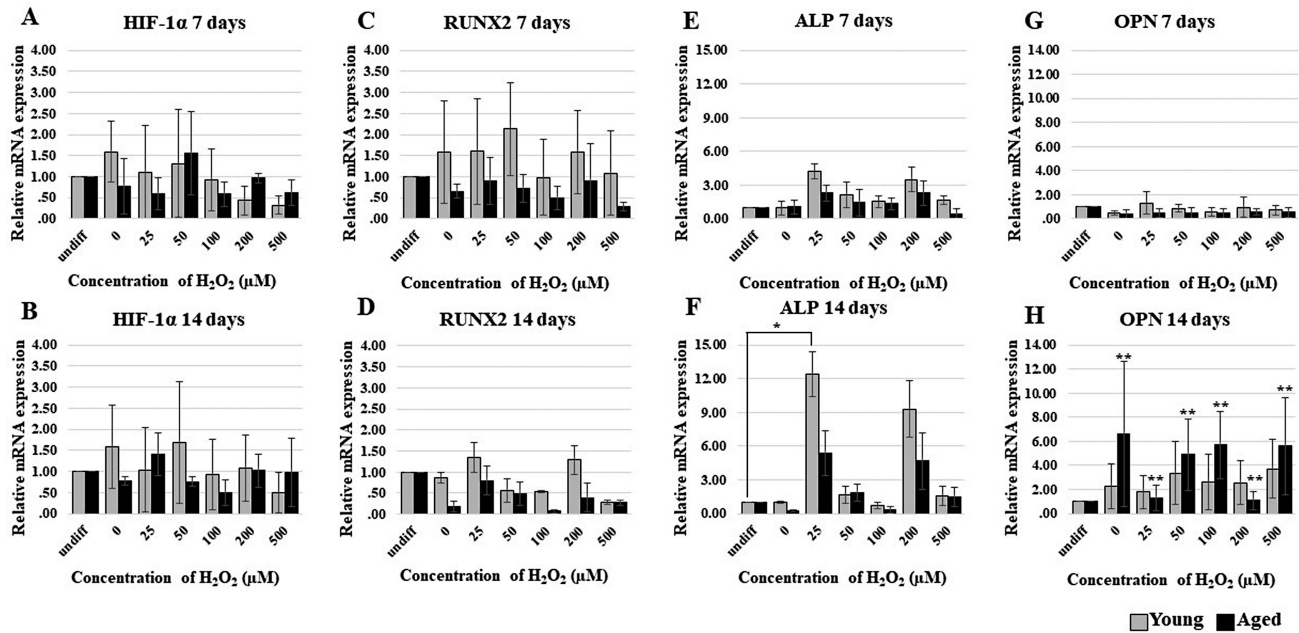


Fig. 5 Gene expression of (A) hypoxia-inducible factor-1 alpha (HIF-1α) on day 7, (B) HIF-1α on day 14, (C) runt-related transcription factor 2 (RUNX2) on day 7, and (D) RUNX2 on day 14. The expressions of young and aged group were not different for all concentration ($p \geq 0.05$, $n = 3$). (E) Alkaline phosphatase (ALP) on day 7 and (F) ALP on day 14. The expression of ALP at concentration 25 and 0 μM of the young group was statistically significantly different ($p = 0.037$, $n = 3$). (G) Osteopontin (OPN) on day 7 and (H) OPN on day 14. The OPN gene expression between 7 and 14 days was statistically significantly different ($p < 0.05$, $n = 3$).

significant difference in RUNX2 gene expression between the aged and young groups at all concentrations (►Fig. 5C, D). At a concentration of 25 μM on day 14 of the ALP gene, a statistically significant difference was demonstrated in the young group when compared to undifferentiated cells ($p=0.037$, ►Fig. 5F). For OPN, there were significant differences of aged group in all concentrations when comparing day 7 with day 14 ($p < 0.05$, ►Fig. 5G, H); however, there was no significant difference between the aged and young groups at all concentrations.

Discussion

Low-level hypoxic conditions have been studied as transient mechanisms of tissue repair and regeneration. HIF-1 α , a hypoxia-related gene, mediates as an intermediary involved in proliferation, cellular migration, and osteogenic differentiation. The hypoxic pathways can upregulate the VEGF expression via HIF-1 α , which enhances angiogenesis and reoxygenation of this hypoxic environment.^{19–24} Moreover, several pluripotency reprogramming factors, which induce lineage differentiations and commit in embryonic development such as Sox2, Oct4, and Nanog, have been suggested that they can exhibit transiently upregulation in response to ischemic sites in the induced pluripotent stem cell.^{26,27} Therefore, low-level hypoxic conditions might play a role in stemness maintenance, proliferation, and differentiation.

With low concentrations of 25 and 50 μM , aged PDL cells demonstrated cytotoxicity, cellular migration, and osteogenic differentiation similar to those of the young group. However, high concentrations of 200 and 500 μM seemed to cause damage and impair the activities of PDL cells. Our results were similar to those of previous studies, which showed that PDL cells and gingival fibroblasts preferred low hypoxic conditions, which could enhance proliferation, osteogenic differentiation,^{21–24} and type I collagen production²⁸ via HIF-1 α in those cells. However, aged PDL cells have not yet been evaluated; our study concluded that a low concentration of H_2O_2 may promote cellular activities, but not to the level observed in the young group. Due to oxygen deprivation caused by accumulated ROS, which frequently occurs during the aging process, the hypoxia-induced mechanism may be very active in aged cells. Therefore, the regeneration potential of aged cells could be comparable to that of younger cells.

To evaluate the aging process in PDL cells, a cellular senescent assay was initially performed in all groups. The senescence-associated marker β -galactosidase was detectable only in the aged group. β -galactosidase is a glycoside hydrolase enzyme that can catalyze the hydrolysis of β -galactosidase into monosaccharides only in senescent cells. Hence, the senescence of PDL cells from aged participants could be confirmed, but senescence was absent in the control and young groups.

In cytotoxicity assays, the release of LDH in the control, young, and aged groups was not significantly different among groups at all concentrations, but the results demonstrated a larger increase in cytotoxicity at 200 and 500 μM . Therefore, when compared to the cellular environment

generated during aging processes, increasingly severe hypoxia in cells contributes to high ROS levels and is harmful to cells and their activities. The use of a cell therapy technique with cells from an aged subject should be the primary determinant of whether there is a low hypoxic condition, and the use of aged cells with a low level of hypoxia could be effective for periodontal regeneration. Cellular tolerance to H_2O_2 varies by cell type and exposure time. Ji et al found that if the concentration of H_2O_2 was not more than 25 μM when neuroblastic cells were exposed for 6 hours, it did not affect cell cytotoxicity. Meanwhile, 50, 100, and 200 μM concentrations induced cell death by 7, 15, and 16%, respectively, compared to the control.²⁹ Moreover, previous studies found that untreated endothelial cells or PDL cells can release LDH equal to 30 to 33% cytotoxicity at 24 hours.^{30,31} LDH release from our study was approximately 8 to 30% with concentration-dependent trend, thus results were similar to those of a previous study that demonstrated the LDH release was time- and concentration-dependent.³² Wang et al found that neuronal cells can tolerate H_2O_2 concentrations up to 100 μM for 48 hours,³³ and Potier et al demonstrated that the cell cytotoxicity of mesenchymal cells is clearly decreased after 120 hours of hypoxic culture.³⁴ Thus, tolerance to oxygen-deprived conditions is dependent on the cells and the duration of the experiments.

Cellular migration of PDL cells at 25 and 50 μM were the appropriate concentrations that tended to promote *in vitro* wound closure. The young and aged groups exhibited a similar percentage of wound closure, which implied that aged and young PDL cells under low levels of hypoxia could be used and that they had similar tendencies during wound closure. Aziz et al demonstrated that concentrations of H_2O_2 at 6.25 and 50 μM are the best concentrations to promote cell migration at 24 and 48 hours.³⁵ On the other hand, Iida et al found that hypoxic conditions enhanced the proliferation of human dental pulp cells if they were exposed to hypoxic conditions for more than 2 days.³⁶ This evidence indicated that a longer exposure period may enhance the cellular migration potential more than a shorter period. However, this study examined cultured PDL cells for only 2 days because long-term culture may conceal cellular migration instead of cell proliferation. Moreover, using H_2O_2 -treated cells may induce more cell detachment, especially at high concentrations; thus, the longer exposure time may be a limitation for the cellular migration assay.

According to osteogenic differentiation, mineralization usually starts at approximately 7 days. Hence, we performed ARS at 7 and 14 days to detect mineralization. Our ARS results and their quantification demonstrated that mineralization at both 7 and 14 days was not different between the young group and the aged group. When analyzing osteogenic gene expression, RUNX2, early markers of osteogenesis and differentiation, showed their highest expression at 25 to 50 μM on day 7 in both aged and young groups. RUNX2 is highly expressed at 7 days and gradually decreases in expression at 14 days.³⁷ At 14 days, the aged group showed higher mineralization, as indicated by ARS, than the young group; according to Grynpas, mineralization shifts toward higher

mineralization during aging.³⁸ In addition, the correlation of idiopathic osteosclerosis of the jaw and carotid artery calcification is related to increasing age.³⁹ However, even though the aged group exhibited high mineralization by ARS, RUNX2 and ALP expression levels in the young group were higher than those in the aged group. Although the concentration of H₂O₂ was not significantly affected when osteogenic differentiation was induced, the results of all osteogenic genes showed that the most suitable concentration was 25 to 50 μM. OPN is highly expressed at 14 to 21 days as an early to intermediate marker of osteogenic differentiation. Both the young group and the aged group showed the same tendency, with a very clear OPN expression at 14 days in both groups. Moreover, Hung et al found that a short period of treatment with hypoxic conditions did not clearly enhance the osteogenic differentiation potential and other evidence suggested that differentiation was promoted under long-term hypoxia.^{21,40} This situation resembles osteogenic differentiation in bone marrow, which lacks oxygen, and is appropriate for osteogenic differentiation of bone marrow stem cells.^{41,42}

To investigate the expression of genes related to hypoxic conditions and osteogenic differentiation, the expression of HIF-1α was detected. HIF-1α, a transcription factor, is induced under hypoxic conditions. Under normoxic conditions, the production of HIF is proportional to its breakdown, which maintains small amounts of HIF-1α within the cells. However, under hypoxic conditions, HIF-1α levels are increased within the cell. Thus, HIF-1α was suggested to be a linking factor that may play a key role in inducing the overexpression of angiogenesis-related factors and contribute to osteogenic differentiation.^{21–24,43}

In our experiment, hypoxia chemically induced by H₂O₂ had different effects at different concentrations. Concentrations of 25 and 50 μM were potentially appropriate for cellular migration and osteogenic differentiation. Although the trend was not statistically significant, our results suggested that a low level of hypoxia may enhance the potential for cellular migration and osteogenic differentiation. Additionally, the aging process, which is always prone to hypoxic conditions, was examined in this study, and it was discovered that PDL cells from aged participants might have been used for osteogenic differentiation for periodontal treatment by periodontal regeneration. Thus, periodontal regeneration in aged individuals could be further investigated to identify approaches for regenerative cell-based therapy. Further study is needed for *in vivo* analysis and investigations of HIF-1α-related mechanisms should be performed in aged PDL cells.

Conclusion

Low concentrations of hypoxic conditions could potentially promote the cellular migration and osteogenic differentiation of aged PDL cells comparable to younger cells, revealing a novel opportunity for using aged PDL cells from an aged individual when performing cell-based therapy for periodontal regeneration. Aging is not a contraindication for using autogenous cells sources.

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Conflict of interest

None declared.

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References

- Hajishengallis G, Chavakis T, Lambris JD. Current understanding of periodontal disease pathogenesis and targets for host-modulation therapy. *Periodontol 2000* 2020;84(01):14–34
- Cekici A, Kantarci A, Hasturk H, Van Dyke TE. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontol 2000* 2014;64(01):57–80
- Swoboda JR, Kiyak HA, Darveau R, Persson GR. Correlates of periodontal decline and biologic markers in older adults. *J Periodontol* 2008;79(10):1920–1926
- Yu YH, Kuo HK, Lai YL. The association between serum folate levels and periodontal disease in older adults: data from the National Health and Nutrition Examination Survey 2001/02. *J Am Geriatr Soc* 2007;55(01):108–113
- Chung HY, Lee EK, Choi YJ, et al. Molecular inflammation as an underlying mechanism of the aging process and age-related diseases. *J Dent Res* 2011;90(07):830–840
- Fransson C, Mooney J, Kinane DF, Berglundh T. Differences in the inflammatory response in young and old human subjects during the course of experimental gingivitis. *J Clin Periodontol* 1999;26(07):453–460
- Norderyd O, Hugoson A, Grusovin G. Risk of severe periodontal disease in a Swedish adult population. A longitudinal study. *J Clin Periodontol* 1999;26(09):608–615
- Ogawa H, Yoshihara A, Hirotsomi T, Ando Y, Miyazaki H. Risk factors for periodontal disease progression among elderly people. *J Clin Periodontol* 2002;29(07):592–597
- Renvert S, Persson GR. Treatment of periodontal disease in older adults. *Periodontol 2000* 2016;72(01):108–119
- Trombelli L, Rizzi A, Simonelli A, Scapoli C, Carrieri A, Farina R. Age-related treatment response following non-surgical periodontal therapy. *J Clin Periodontol* 2010;37(04):346–352
- Han NY, Hong JY, Park JM, et al. Label-free quantitative proteomic analysis of human periodontal ligament stem cells by high-resolution mass spectrometry. *J Periodontol Res* 2019;54(01):53–62
- Xing Y, Zhang Y, Wu X, Zhao B, Ji Y, Xu X. A comprehensive study on donor-matched comparisons of three types of mesenchymal stem cells-containing cells from human dental tissue. *J Periodontol Res* 2019;54(03):286–299
- Bryant SR, Zarb GA. Osseointegration of oral implants in older and younger adults. *Int J Oral Maxillofac Implants* 1998;13(04):492–499
- Yeo EJ. Hypoxia and aging. *Exp Mol Med* 2019;51(06):1–15
- Valli A, Harris AL, Kessler BM. Hypoxia metabolism in ageing. *Aging (Albany NY)* 2015;7(07):465–466
- Kim JH, Song SY, Park SG, Song SU, Xia Y, Sung JH. Primary involvement of NADPH oxidase 4 in hypoxia-induced generation of reactive oxygen species in adipose-derived stem cells. *Stem Cells Dev* 2012;21(12):2212–2221
- Busletta C, Novo E, Valfrè Di Bonzo L, et al. Dissection of the biphasic nature of hypoxia-induced mitogenic action in bone

- marrow-derived human mesenchymal stem cells. *Stem Cells* 2011;29(06):952–963
- 18 Li S, Deng Y, Feng J, Ye W. Oxidative preconditioning promotes bone marrow mesenchymal stem cells migration and prevents apoptosis. *Cell Biol Int* 2009;33(03):411–418
 - 19 De Barros S, Dehez S, Arnaud E, et al. Aging-related decrease of human ASC angiogenic potential is reversed by hypoxia preconditioning through ROS production. *Mol Ther* 2013;21(02):399–408
 - 20 Kim JH, Park SG, Song SY, Kim JK, Sung JH. Reactive oxygen species-responsive miR-210 regulates proliferation and migration of adipose-derived stem cells via PTPN2. *Cell Death Dis* 2013;4(04):e588
 - 21 Hung SP, Ho JH, Shih YRV, Lo T, Lee OK. Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. *J Orthop Res* 2012;30(02):260–266
 - 22 Kifune T, Ito H, Ishiyama M, et al. Hypoxia-induced upregulation of angiogenic factors in immortalized human periodontal ligament fibroblasts. *J Oral Sci* 2018;60(04):519–525
 - 23 Ng KT, Li JP, Ng KM, Tipoe GL, Leung WK, Fung ML. Expression of hypoxia-inducible factor-1 α in human periodontal tissue. *J Periodontol* 2011;82(01):136–141
 - 24 Zou D, Han W, You S, et al. In vitro study of enhanced osteogenesis induced by HIF-1 α -transduced bone marrow stem cells. *Cell Prolif* 2011;44(03):234–243
 - 25 Nagatomo K, Komaki M, Sekiya I, et al. Stem cell properties of human periodontal ligament cells. *J Periodontol Res* 2006;41(04):303–310
 - 26 Jung GA, Kim JA, Park HW, et al. Induction of Nanog in neural progenitor cells for adaptive regeneration of ischemic brain. *Exp Mol Med* 2022;54(11):1955–1966
 - 27 De Miguel MP, Alcaina Y, de la Maza DS, Lopez-Iglesias P. Cell metabolism under microenvironmental low oxygen tension levels in stemness, proliferation and pluripotency. *Curr Mol Med* 2015;15(04):343–359
 - 28 Morimoto C, Takedachi M, Kawasaki K, et al. Hypoxia stimulates collagen hydroxylation in gingival fibroblasts and periodontal ligament cells. *J Periodontol* 2021;92(11):1635–1645
 - 29 Ji G, Lv K, Chen H, et al. MiR-146a regulates SOD2 expression in H2O2 stimulated PC12 cells. *PLoS One* 2013;8(07):e69351
 - 30 Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Mol Ther* 2005;11(06):990–995
 - 31 Chen Z, Patwari M, Liu D. Cytotoxicity of orthodontic temporary anchorage devices on human periodontal ligament fibroblasts in vitro. *Clin Exp Dent Res* 2019;5(06):648–654
 - 32 Ye J, Tang X, Long Y, Chu Z, Zhou Q, Lin B. The effect of hypoxia on the proliferation capacity of dermal papilla cell by regulating lactate dehydrogenase. *J Cosmet Dermatol* 2021;20(02):684–690
 - 33 Wang G, Chen S, Shao Z, et al. Metformin alleviates hydrogen peroxide-induced inflammation and oxidative stress via inhibiting P2 \times 7R signaling in spinal cord tissue cells neurons. *Can J Physiol Pharmacol* 2021;99(08):768–774
 - 34 Potier E, Ferreira E, Andriamanalijaona R, et al. Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. *Bone* 2007;40(04):1078–1087
 - 35 Aziz NSM, Stanslas J, Kadir NH ABD. Hydrogen peroxide promotes human skin fibroblast cells migration and proliferation at lower dose via VEGF-alpha and TGF-alpha gene expression. *Malays Appl Biol* 2020;49:261–266
 - 36 Iida K, Takeda-Kawaguchi T, Tezuka Y, Kunisada T, Shibata T, Tezuka K. Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells. *Arch Oral Biol* 2010;55(09):648–654
 - 37 Pitkänen S. *In Vitro* and *In Vivo* Osteogenesis and Vasculogenesis in Synthetic Bone Grafts [thesis]. Tampere, Finland: Tampere University; 2020:120p
 - 38 Grynepas M. Age and disease-related changes in the mineral of bone. *Calcif Tissue Int* 1993;53(Suppl 1):S57–S64
 - 39 Kaplan SS, Kaplan T, Sezgin GP. Assessment of the frequency and correlation of carotid artery calcifications and pulp stones with idiopathic osteosclerosis using digital panoramic radiographs. *Clin Exp Health Sci* 2021;11:342–347
 - 40 Hung SC, Pochampally RR, Hsu SC, et al. Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo. *PLoS One* 2007;2(05):e416
 - 41 Chow DC, Wenning LA, Miller WM, Papoutsakis ET. Modeling pO₂ distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys J* 2001;81(02):685–696
 - 42 Ferrer A, Roser CT, El-Far MH, et al. Hypoxia-mediated changes in bone marrow microenvironment in breast cancer dormancy. *Cancer Lett* 2020;488:9–17
 - 43 Hubbi ME, Semenza GL. Regulation of cell proliferation by hypoxia-inducible factors. *Am J Physiol Cell Physiol* 2015;309(12):C775–C782