jdhs
Journal of Dental Hygiene Science

J Dent Hyg Sci Vol. 23, No. 4, 2023, pp.264-270
https://doi.org/10.17135/jdhs.2023.23.4.264

RESEARCH ARTICLE

Influence of 10-Methacryloyloxydecyl Dihydrogen Phosphate on Cellular Senescence in Osteoblast-Like Cells

Ju Yeon Ban[1] and Sang-Im Lee[2,†]

[1]Department of Dental Pharmacology, College of Dentistry, Dankook University, Cheonan 31116, [2]Department of Dental Hygiene, College of Health Science, Dankook University, Cheonan 31116, Korea

Background: Resin-based dental materials release residual monomers or other substances from incomplete polymerization into the oral cavity, thereby causing adverse biological effects on oral tissue. 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP), an acidic monomer containing dihydrogen phosphate and methacrylate groups, is the most commonly used component of resin-based dental materials, such as restorative composite resins, dentin adhesives, and resin cements. Although previous studies have reported the cytotoxicity and biocompatibility in various cultured cells, the effects of resin monomers on cellular aging have not been reported to date. Therefore, this study aimed to investigate the effects of the resin monomer 10-MDP on cellular senescence and inflamm-aging in vitro.

Methods: After stimulation with 10-MDP, MC3T3-E1 osteoblast-like cells were examined for cell viability by WST-8 assay and reactive oxygen species (ROS) production by flow cytometry. The protein and mRNA levels of molecular markers of aging were determined by western blotting and RT-PCR analysis, respectively.

Results: Treatment with 0.05 to 1 mM 10-MDP for 24 hours reduced the survival of MC3T3-E1 cells in a concentration-dependent manner. The intracellular ROS levels in the 10-MDP-treated experimental group were significantly higher than those in the control group. 10-MDP at a concentration of 0.1 mM increased p53, p16, and p21 protein levels. Additionally, an aging pattern was observed with blue staining due to intracellular senescence-associated beta-galactosidase activity. Treatment with 10-MDP increased the levels of tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-8, however their expression was decreased by mitogen-activated-protein-kinase (MAPK) inhibitors.

Conclusion: Taken together, these results suggest that the exposure of osteoblast-like cells to the dental resin monomer 10-MDP, increases the level of cellular senescence and the inflammatory response is mediated by the MAPK pathway.

Key Words: 10-Methacryloyloxydecyl dihydrogen phosphate, Cellular senescence, Cytotoxicity, Dental resin monomer

Introduction

1. Background

Oral pathologies are primarily infectious diseases related to the immune system. Altered immune response due to cell damage in the dental pulp and surrounding tissues lead to a chronic, mild inflammatory state called inflamm-aging[1)]. During inflammatory aging, the levels of inflammatory cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α increase. These cytokines enhance immune activity, resulting in dysfunction of dendritic cells, neutrophils, macrophages, and fibroblasts[2)]. The accumulation of senescent cells enhances aging by causing chronic inflammation and metabolic disorders. Excessive secretion of inflammatory mediators by senescent cells with an increased senescence-associated secretion phenotype (SASP) further reduces the efficiency of the immune response[3)]. The cytotoxic cells that accumulate in the oral tissues secrete enzymes, such as matrix metalloproteinases, serine proteases, and cathepsins, which are involved the

Received: October 13, 2023, **Revised:** November 7, 2023, **Accepted:** November 10, 2023 eISSN 2233-7679

†**Correspondence to:** Sang-Im Lee, *https://orcid.org/0000-0003-2635-6876*
Department of Dental Hygiene, College of Health Science, Dankook University, 119 Dandae-ro, Dongnam-gu, Cheonan 31116, Korea
Tel: +82-41-550-1492, E-mail: hanjumuck@dankook.ac.kr

degradation of the extracellular matrix. As a result, more matrix is exposed to these enzymes, resulting in incresed oral tissue destruction[4].

Composite resins and dental adhesives are commonly used in clinical dentistry to treat tooth damage caused by dental caries, fractures, and erosion. Common dental composite resins and adhesives for tooth restoration include monomers such as 2-hydroxyethyl methacrylate (HEMA), tetraethylene glycol dimethacrylate (TEGDMA), and bisphenol glycidyl methacrylate (Bis-GMA)[5]. In particular, 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP) is the main ingredient of multiple resin-based dental materials such as adhesive systems and bonding resin cements, and it is the most widely used acidic monomer[6]. Although various dental materials containing acidic resin monomers are commonly used in dental clinics, research on their toxicity and effects on physiological functions in the oral environment is lacking. Therefore, this study aimed to investigate the inflamm-aging mechanism of the commonly used acidic resin monomer 10-MDP.

2. Objectives

There is a lack of understanding of the cellular aging mechanisms to involved in tissue destruction caused by inflammatory processes in oral diseases. Therefore, the objective of the present study was to investigate the cytotoxic effect of 10-MDP, which is a functional monomer widely used in dental materials, on osteoblasts-like cells and to identify the signaling pathways involved in the cellular senescence.

Materials and Methods

1. Study design

1) Cell culture and cytotoxicity test

MC3T3-E1 osteoblast-like cells were cultured in α-MEM (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% inactivated fetal bovine serum (Gibco) at 37°C in 5% CO_2. After overnight incubation, the cells were seeded into a culture dish and incubated with various concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mM) of 10-MDP (Watson International Ltd., Kunshan, China). For cell culture experiments, 10-MDP was directly dissolved in the cell culture medium. Cell viability was measured by WST-8 assays (MediFab, Seoul, Korea), according to the manufacturer's instructions. The fluorescence signal due to the oxidation of CM-H_2DCFDA dye (Molecular Probes, Eugene, OR, USA) was used to measure the level of intracellular free radicals such as reactive oxygen species (ROS). After treatment with the resin monomer, MC3T3-E1 cells were washed with phosphate-buffered saline (PBS) and loaded with 10 μM CM-H_2DCFDA PBS for 15 minutes at 37°C in the dark. The dichlorofluorescein (DCF) fluorescence of the cells in each test group was assessed using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA), with excitation and emission wavelengths of 480 and 530 nm, respectively. The data are presented as mean±standard deviation in triplicate experiments.

2) Detection of molecular markers of aging

Cells were cultured with 0.1 mM 10-MDP for 24 hours. Proteins were extracted from harvested cells using the recommended pro-prep protocol (iNtRON Biotechnology, Seongnam, Korea). The protein from each sample was subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel, electrophoresis and then transferred to a membrane by electroblotting. The membranes were incubated for four hours at room temperature (RT) with primary antibodies against p53, p16, p21, and β-actin (Cell Signaling Technology, Danvers, MA, USA), followed by secondary antibody for one hour at RT. Immunoreactive bands were visualized using a chemiluminescence system (Bio-Rad, Hercules, CA, USA). Signals were detected using Micro-Chemi (DNR Bio-Imaging, Modi'in-Maccabim-Re'ut, Israel). Senescence-associated beta-galactosidase (SA-β-gal) activity was determined using a SA-β-gal staining kit (Cell Signaling Technology) according to the manufacturer's instructions. Senescent cells were identified as blue-stained cells by standard light microscopy at 100× magnification. The representative data is representative of three independent experiments.

3) Gene expression analysis by RT-PCR

To investigate the effects of 0.1 mM 10-MDP on molecular markers of aging in MC3T3-E1 cells, mRNA levels were measured by reverse transcriptase-polymerase chain

reaction (RT-PCR). Cells were pretreated with the JNK inhibitor SP600125 (5 μM; Calbiochem, La Jolla, CA, USA), ERK1/2 inhibitor U0126 (5 μM; Calbiochem), or p38 inhibitor SB203580 (10 μM; Calbiochem) for 1 hour, and then treated with 10-MDP for 24 hours. Total RNA was extracted using TRIzol® reagent (Welgene Inc., Daegu, Korea), and cDNA was prepared using AccuPower® RT PreMix (Bioneer, Daejeon, Korea). The RT-generated DNA (2～5 μl) was then amplified using AccuPower® PCR PreMix (Bioneer). The following primers were used: TNF-α, forward (F): 5'-CCCAAGGCTATAAAGCGG-3', reverse (R): 5'-CCCAAGGGCTATAAGGCGG-3'; IL-1β, F: 5'-TGCCACCTTTTGACAGTGATG-3', R: 5'-GGAG CCTGTAGTGCAGTTGT-3'; IL-6, F: 5'-GCCTTCTTG GGACTGATGCT-3', R: 5'-TGTGACTCCAGCTTATCT CTTGG-3'; IL-8, F: 5'-TGCTTTTGGCTTTGCGTTGA-3', R: 5'-GTCAGAACGTGGCGGTATCT-3'; glyceralde-hyde-3-phosphate dehydrogenase (GAPDH), F: 5'-GCAT CTTCTTGTGCAGTGCC-3', R: 5'-TACGGCCAAATC CGTTCACA-3'. The PCR thermocycling conditions were: 94°C for 30 seconds, followed by 30 cycles of denatu-ration at 95°C for 15 seconds and annealing at 62°C for 30 seconds. Representative data were obtained from three independent experiments.

Results

1. Cytotoxicity effects of the resin monomer 10-MDP

To determine whether 10-MDP affected the viability of MC3T3-E1 cells, we assessed its cytotoxicity by WST-8 assay (Fig. 1A). 10-MDP exerted concentration-dependent (0.05～1 mM) cytotoxicity. 10-MDP exposure resulted in cell viabilities of 95% and 93% at 0.05 and 0.1 mM, res-pectively. Concentrations of 10-MDP up to 0.1 mM did not exert significant cytotoxicity, while 0.2 mM resulted in a relatively high level of cytotoxicity. Because ROS are known to contribute to cellular senescence[4], we the ROS levels in MC3T3-E1 cells exposed to 10-MDP. As shown in Fig. 1B, as the 10-MDP concentration increased the fluorescence of DCF in MC3T3-E1 cells increased, indi-cating that intracellular ROS levels were higher than those of the control groups. Therefore, based on the results of both experimental methods, the minimal toxic concent-ration of 10-MDP (0.1 mM) was used.

2. Involvement of mitogen-activated-protein-kinase pathways in 10-MDP-induced inflamm-aging responses

Because cell senescence or apoptosis occur as a result of cell cycle arrest, we investigated the expression of cell cycle-related molecules. Exposure of MC3T3-E1 cells to

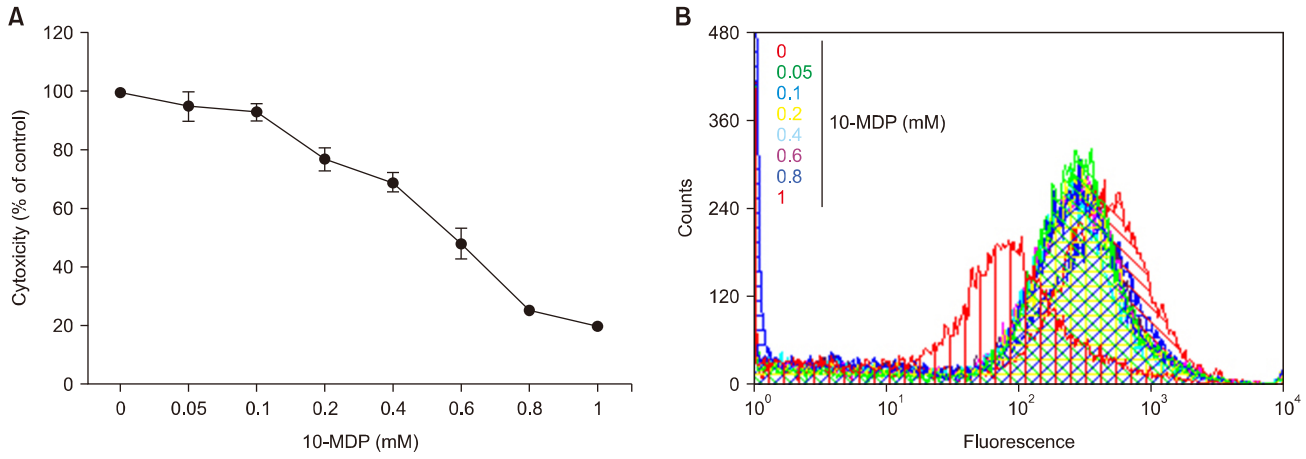

Fig. 1. Effects of 10-MDP on cytotoxicity and reactive oxygen species (ROS) production in MC3T3-E1 osteoblast-like cells. Cells were treated with 10-MDP (0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mM) for 24 hours. (A) Cell viability was estimated by WST-8. (B) Production of intracellular ROS is presented as the mean fluorescence intensity of DCF determined by flow cytometry. The values are expressed as mean±standard deviation from triplicate experiments. 10-MDP: 10-Methacryloyloxydecyl dihydrogen phosphate.

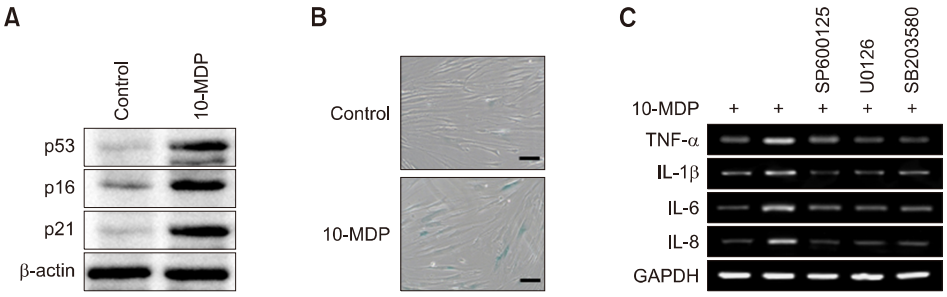

Fig. 2. Effects of 10-MDP on signal transduction pathways. Cells were cultured with or without 0.1 mM 10-MDP for 24 hours. (A) Expression of cell cycle-related protein. (B) Senescence-associated β-galactosidase (SA-β-gal) staining. Scale bar=50 μm (C) Production of senescence-associated cytokines. Cells were pretreated with MAPK inhibitors (JNK inhibitor SP600125, ERK1/2 inhibitor U0126, or p38 inhibitor SB203580) for 1 hour and then treated with 0.1 mM 10-MDP for 24 hours. Data of representative of three independent experiments are shown. 10-MDP: 10-Methacryloyloxydecyl dihydrogen phosphate, TNF: tumor necrosis factor, IL: interleukin.

100 μM 10-MDP significantly increased the protein expression levels of the cell cycle-related senescence markers p53, p16, and p21 (Fig. 2A). When SA-β-gal staining was performed to confirm the degree of cellular senescence, there was a clear increase in SA-β-gal positive cells (Fig. 2B). To confirm the role of mitogen-activated-protein-kinase (MAPK) in senescence-associated cytokine production in 10-MDP-treated MC3T3-E1 cells, the cells were pretreated with JNK, ERK1/2, p38 inhibitors. Treatment with 0.1 mM 10-MDP induced secretion of TNF-α, IL-1β, IL-6 and IL-8, and their expression was suppressed by MAPK (JNK, ERK1/2, and p38) inhibitors (Fig. 2C).

Discussion

1. Interpretation

Dental resin-based materials are widely used in dental resin restorations, adhesion of prosthetics, and denture manufacturing, and excessive use of composite resin cement plays an important role in the development of gingivitis and periodontitis[7]. Therefore, important to evaluate the cytotoxicity of resin monomers toward bone cells. In this study, we found that cellular senescence, which is a state of cellular damage, was activated in osteoblasts in response to the cytotoxicity induced by dental resin monomers. To our knowledge, this is the first study to demonstrate that 10-MDP-induced cellular senescence activation is associated with the MAPK pathway in osteoblasts.

2. Key results and comparison

Inflammation is a reaction that occurs when an infection or wound occurs in a tissue to protect it from damage to return it and return the tissue to its normal state. Chronic inflammation is a low-grade, persistent inflammatory condition that occurs when acute inflammation does not heal well or when inflammation occurs repeatedly in the same area, and the reaction to stimulation or damage progresses slowly[1]. Chronic inflammation of the oral cavity due to periodontal disease has been reported to lead to damage and dysfunction of tissues and organs throughout the body (a direct or indirect cause of several age-related diseases such as periodontitis, cardiovascular disease, type 2 diabetes, cancer, and neurodegenerative disorders)[8,9].

TEGDMA and HEMA monomers can cause cytotoxicity and allergic reaction in gingival cells[10,11]. Previous studies have reported that the negative effects of minimally toxic concentrations of TEGDMA and HEMA on the differentiation of dental pulp cells into odontoblasts can reduce the mineralization process, which is an important biological response of pulp tissue[6,12]. Bis-GMA is an endocrine disruptor, and animal studies have shown that it has reproductive, developmental and systemic toxic effects even at low doses[11,13]. These monomers have been shown to induce cellular toxicity and apoptosis in human dental pulp cells through intracellular glutathione depletion and ROS production. In our study, to investigate the effect of 10-MDP on periodontal inflamm-aging, MC3T3-E1 osteoblast-like cells were treated with 0.05～1 mM 10-MDP. Kim et al.[6] reported that 10-MDP inhibited human pulp cell prolifera-

tion and increased inflammatory response in a concentration-dependent manner in the range of 0.25 to 0.2 mM. Compared with previous studies, 10-MDP result in a similar level of cytotoxicity in the MC3T3-E1 osteoblast-like cells used in this study (Fig. 1A).

A previous study reported that periodontal tissue continuously releases ROS under chronic inflammatory conditions, which can damage DNA and cell structure in neighboring cells[4]. Damage from free radicals accumulates, causing mutations and tissue damage that trigger aging. Chronic inflammation can also lead to the accumulation of senescent cells, which stop dividing but remain active to produce inflammatory substances[14]. Senescent cells accelerate the aging process by damaging the surrounding tissues and by producing signals that impede cell function[15]. The level of ROS increased in 10-MDP-treated cells (Fig. 1B), which is consistent with previous evidence that dental resin monomers cause cytotoxicity in periodontal cell[16].

Cellular senescence is a state of permanent cell cycle arrest and contributes to a pro-inflammatory environment, resulting in tissue dysfunction and reduced regenerative capacity. As a result of the cell cycle arrest, the cells become senescent, transform into cancer cells, and undergo apoptosis. The aging marker p53 accumulates in damaged and senescent cells[17]. p16 is a marker of apoptosis independent of p53 and is a known aging marker, whereas p21 is a marker of apoptosis dependent on p53 and has been reported to be an aging marker that promotes aging[18]. Consistent with previous results, our findings revealed that the expression levels of the well-known senescence marker of p53, p16, and p21 were increased in the 10-MDP group, indicating that the resin monomer caused cellular senescence (Fig. 2A). These results are consistent with the microscopy results and further consistent with increased β-gal activity (Fig. 2B).

However, it is unclear how cellular senescence is activated mechanistically by 10-MDP. Recent studies have revealed a key role of MAPK-dependent aging in periodontitis[19]. Our previous findings showed that autophagy plays an important role in cellular senescence[20]. Therefore, we investigated the role of MAPK signaling in 10-MDP-induced cellular senescence. Senescent fibroblasts have been reported to release several inflammatory factors, including IL-1α, IL-1β, IL-6, and IL-17[14,21]. By confirming the SASP signature, we found that IL-1β, TNF-α, and IL-6 were significantly increased in 10-MDP-treated senescent cells (Fig. 2C). We also found that osteoblast senescence was rescued by a MAPK inhibitor, suggesting that the senescence induced by 10-MDP is regulated by MAPK. Recent evidence that senescent osteocytes accumulate in murine alveolar bone and display p38 MAPK activation in vivo[19] lends support to our findings are plausible. Therefore, the senescence of osteoblasts in periodontal tissue has been suggested to be evidence of inflammatory cascades.

3. Limitations

Our study had several limitations. This study relied on only a limited number of molecular markers to confirm cellular senescence, and the immune network with surrounding cells, which is important in inflamm-aging, was not investigated. Furthermore, in vivo experiments are insufficient to confirm details of the underlying mechanism. Nevertheless, our findings provide a scientific basis for novel molecular targeting of inflamm-aging for dental monomer-related cellular damage.

4. Conclusion

Collectively, our findings indicate that exposure to dental resin monomers results in cell damage and oxidative stress, thereby leading to cellular aging. Additionally, our results indicate that 10-MDP-mediated cellular senescence in osteoblasts is regulated by the MAPK signaling pathway.

Notes

Conflict of interest

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

Ethical approval

Not applicable.

Author contributions

Conceptualization: Sang-Im Lee. Data acquisition: Ju Yeon Ban and Sang-Im Lee. Formal analysis: Ju Yeon Ban and Sang-Im Lee. Funding: Sang-Im Lee. Supervision:

Sang-Im Lee. Writing - original draft: Ju Yeon Ban and Sang-Im Lee. Writing - review & editing: Sang-Im Lee. All authors have read and agreed to the published version of the manuscript.

ORCID

Ju Yeon Ban, *https://orcid.org/0000-0003-1992-5188*
Sang-Im Lee, *https://orcid.org/0000-0003-2635-6876*

Funding

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2022R1F1A1076563).

Acknowledgements

None.

Data availability

The data will be available by corresponding authors upon genuine request.

References

1. Franceschi C, Campisi J: Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci 69 Suppl 1: S4-S9, 2014. https://doi.org/10.1093/gerona/glu057
2. Freund A, Orjalo AV, Desprez PY, Campisi J: Inflammatory networks during cellular senescence: causes and consequences. Trends Mol Med 16: 238-246, 2010. https://doi.org/10.1016/j.molmed.2010.03.003
3. Hearps AC, Martin GE, Angelovich TA, et al.: Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. Aging Cell 11: 867-875, 2012. https://doi.org/10.1111/j.1474-9726.2012.00851.x
4. Ikegami K, Yamashita M, Suzuki M, et al.: Cellular senescence with SASP in periodontal ligament cells triggers inflammation in aging periodontal tissue. Aging (Albany NY) 15: 1279-1305, 2023. https://doi.org/10.18632/aging.204569
5. Cramer NB, Stansbury JW, Bowman CN: Recent advances and developments in composite dental restorative materials. J Dent Res 90: 402-416, 2011. https://doi.org/10.1177/0022034510381263
6. Kim EC, Park H, Lee SI, Kim SY: Effect of the acidic dental resin monomer 10-methacryloyloxydecyl dihydrogen phosphate on odontoblastic differentiation of human dental pulp cells. Basic Clin Pharmacol Toxicol 117: 340-349, 2015. https://doi.org/10.1111/bcpt.12404
7. Kraus D, Wolfgarten M, Enkling N, et al.: In-vitro cytocompatibility of dental resin monomers on osteoblast-like cells. J Dent 65: 76-82, 2017. https://doi.org/10.1016/j.jdent.2017.07.008
8. Ebersole JL, Graves CL, Gonzalez OA, et al.: Aging, inflammation, immunity and periodontal disease. Periodontol 2000 72: 54-75, 2016. https://doi.org/10.1111/prd.12135
9. Watanabe S, Kawamoto S, Ohtani N, Hara E: Impact of senescence-associated secretory phenotype and its potential as a therapeutic target for senescence-associated diseases. Cancer Sci 108: 563-569, 2017. https://doi.org/10.1111/cas.13184
10. Chang HH, Guo MK, Kasten FH, et al.: Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. Biomaterials 26: 745-753, 2005. https://doi.org/10.1016/j.biomaterials.2004.03.021
11. Chang MC, Lin LD, Chan CP, et al.: The effect of BisGMA on cyclooxygenase-2 expression, PGE2 production and cytotoxicity via reactive oxygen species- and MEK/ERK-dependent and -independent pathways. Biomaterials 30: 4070-4077, 2009. https://doi.org/10.1016/j.biomaterials.2009.04.034
12. Bakopoulou A, Leyhausen G, Volk J, et al.: Effects of HEMA and TEDGMA on the in vitro odontogenic differentiation potential of human pulp stem/progenitor cells derived from deciduous teeth. Dent Mater 27: 608-617, 2011. https://doi.org/10.1016/j.dental.2011.03.002
13. Chang MC, Chen LI, Chan CP, et al.: The role of reactive oxygen species and hemeoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by BisGMA. Biomaterials 31: 8164-8171, 2010. https://doi.org/10.1016/j.biomaterials.2010.07.049
14. Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA: Age and age-related diseases: role of inflammation triggers and cytokines. Front Immunol 9: 586, 2018.

https://doi.org/10.3389/fimmu.2018.00586
15. Singh T, Newman AB: Inflammatory markers in population studies of aging. Ageing Res Rev 10: 319-329, 2011. https://doi.org/10.1016/j.arr.2010.11.002
16. Schweikl H, Spagnuolo G, Schmalz G: Genetic and cellular toxicology of dental resin monomers. J Dent Res 85: 870-877, 2006. https://doi.org/10.1177/154405910608501001
17. Kim RH, Kang MK, Kim T, et al.: Regulation of p53 during senescence in normal human keratinocytes. Aging Cell 14: 838-846, 2015. https://doi.org/10.1111/acel.12364
18. Zhao P, Yue Z, Nie L, et al.: Hyperglycaemia-associated macrophage pyroptosis accelerates periodontal inflammaging. J Clin Periodontol 48: 1379-1392, 2021. https://doi.org/10.1111/jcpe.13517
19. Aquino-Martinez R, Eckhardt BA, Rowsey JL, et al.: Senescent cells exacerbate chronic inflammation and contribute to periodontal disease progression in old mice. J Periodontol 92: 1483-1495, 2021. https://doi.org/10.1002/JPER.20-0529
20. Jun NR, Jang JH, Lee JY, Lee SI: Autophagy may mediate cellular senescence by nicotine stimulation in gingival fibroblasts. J Dent Hyg Sci 22: 164-170, 2022. https://doi.org/10.17135/jdhs.2022.22.3.164
21. Lee SI: Expression of senescence-associated secretory phenotype in senescent gingival fibroblasts. J Dent Hyg Sci 23: 169-175, 2023. https://doi.org/10.17135/jdhs.2023.23.2.169