Dental Materials Journal 2023; 42(3): 360–367

Localization of senescent cells under cavity preparations in rats and restoration of reparative dentin formation by senolytics

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Reparative dentin formed by dental cavity preparation (DCP) is frequently used in clinical operations and plays a pivotal role in pulp protection. Recent reports have shown that senescent cells induced by various stressors aggravate many diseases. They can be treated using senolytics, which are drugs that selectively eliminate senescent cells. However, the association between DCP, senescent cells, and senolytics remains unclear. In this study, we established a rat model of DCP and analyzed the spatiotemporal localization of senescent cells in the pulp. The results showed that p21- and p16-positive senescent cells appeared mostly around the pulp horn (PH) under DCP. Furthermore, administration of senolytics (dasatinib and quercetin) successfully eliminated these senescent cells, thereby restoring the volume of reparative dentin formation. These data indicate that senescent cells induced by DCP may hamper the formation of reparative dentin. Senescent cells may be targets for the development of new restorative dentistry therapies.

Keywords: Senescence, Senolytics, Cavity preparation, Reparative dentin

INTRODUCTION

Dentin is a hard tissue behind the enamel that protects against external stimuli that threaten the inner pulp tissue[1,2]. Reparative dentin, the third dentin, is formed after the completion of the permanent tooth in response to exogenous stimuli, such as attrition, caries, abrasion, trauma, erosion, or dental cavity preparation (DCP)[3-5]. Additionally, in clinical treatment, when the cavity bottom is close to the pulp, a pulp lining such as $Ca(OH)_2$ is frequently used to block stimuli and enhance the formation of reparative dentin in the pulp[6]. However, there are many clinical cases in which the amount of reparative dentin formed by cavity preparation is insufficient, occasionally resulting in severe pulpitis[7]. Thus, alternative methods to form reparative dentin are still being investigated[8].

In recent years, cellular senescence has attracted considerable attention in various diseases[9-11]. Cellular senescence leading to cell cycle arrest is triggered by DNA damage due to various stressors[12], such as mechanical stress, inflammation, infection, and oxidation[13]. Senescent cells that accumulate in the body secrete various inflammatory proteins called senescence-associated secretory phenotypes (SASPs), which induce inflammation and carcinogenesis in surrounding tissues[14-16]. The components of SASPs, such as inflammatory cytokines, induce mild chronic inflammation, which increases the incidence of atherosclerosis, rheumatoid arthritis, type 2 diabetes, osteoporosis, and Alzheimer's disease[17-21]. However, the cellular behavior and mechanisms of senescent cells after DCP have not been fully elucidated.

Recently, senolytics, which specifically promote cell death in senescent cells by inhibiting upregulated pro-survival mechanisms, have been developed and investigated[22]. Dasatinib (D) and quercetin (Q) were the first to be discovered and are the most studied senolytics[23]. To date, various studies have shown that oral administration of DQ reduces senescent cells and the associated secretion of inflammatory molecules, alleviating physiological dysfunction in the bone[24], fat[25], lung[26], and cardiovascular systems[27].

Based on these findings, we hypothesized that DCP, acting as a stressor, induces senescence, which is associated with the formation of reparative dentin. In the present study, we investigated the appearance and localization of senescent cells in a non-pulp-exposed rat model of DCP. Additionally, we evaluated whether DQ administration alters reparative dentin formation.

MATERIALS AND METHODS

Animal experiments

The 8-week-old male Wistar rats were purchased from SHIMIZU Laboratory (Kyoto, Japan). Animals were housed in an environmentally controlled room. All experimental procedures involving animals were approved by the local ethics committee of Osaka Dental University (approval no. 22-02034).

Cavity preparation and group division

To anesthetize the rats before DCP, each rat was administrated intraperiotoneally with a mixture of midazolam (2 mg/kg; Midazolam Sandoz, Sandoz, Yamagata, Japan), medetomidine hydrochloride (0.15 mg/kg; Domitor, Zenoaq, Fukushima, Japan), and butorphanol tartrate (2.5 mg/kg; Vetorphale, Meiji Seika Pharma, Tokyo, Japan). To prepare a non-pulp-exposed DCP model, the mesial cusp of the maxillary right first

Received Oct 31, 2022: Accepted Nov 28, 2022
doi:10.4012/dmj.2022-245 JOI JST.JSTAGE/dmj/2022-245

molar in each rat was drilled using a straight handpiece with round diamond points (diameter: 0.5 mm; HORICO, Berlin, Germany) under water-cooling (Figs. 1a and b). The cavity was dried using sterile cotton balls with or without indirect pulp capping with $Ca(OH)_2$ (Dycal, Dentsply Sirona, Charlotte, NC, USA). All the cavities were filled with 4-META/MMA-TBB resin (Super-Bond, Sun Medical, Shiga, Japan). In the control group, the first molars on the other side were sealed using 4-META/MMA-TBB resin. The experimental group was divided into three groups: no DCP (control), and DCP with or without indirect pulp cupping with calcium hydroxide ($Ca(OH)_2$) (Fig. 1b). For each experimental group, the pulp horn (PH) and lateral side of the pulp chamber (LPC) were analyzed in the tissue sections (Fig. 1a). The three groups were further divided into two groups, with and without senolytic administration, as described later. The residual dentin thickness was measured on the tissue sections to confirm the establishment of non-exposed pulp (Fig. 1d).

Sample collection

The rats were sacrificed, and the maxillary bones were harvested 1 or 2 weeks after DCP. Decalcification was performed using decalcifying solution B (FUJIFILM Wako Pure Chemical, Osaka, Japan), followed by gradient dehydration with a sucrose solution and freezing. All frozen samples were prepared and stored at −80°C.

Hematoxylin-eosin (H-E) staining and quantitative analysis

For histological analysis, the samples were embedded in Super Cryoembedding Medium (SECTION-LAB, Hiroshima, Japan). Blocks were sliced into 10 µm frozen sections using a cryotome (Leica CM3050S, Leica Biosystems, Richmond, IL, USA) using the Kawamoto method[28]. The sections were subjected to H-E staining to visualize the reparative dentin. H-E sections were observed using a BZ-9000 digital microscope (Keyence, Osaka, Japan). Histomorphometric analysis was performed using Adobe Photoshop Elements (Adobe Systems, San Jose, CA, USA) and ImageJ software (version 2.1.0, U.S. National Institutes of Health, Bethesda, MD, USA). Histological analysis was performed on the PH and LPC. Reparative dentin in the region of interest on the PH and LPC was quantified as the ratio of the area of reparative dentin to the area of the pulp chamber according to the following equation: [(area of reparative dentin/area of pulp chamber)×100].

Immunohistochemistry analysis

Immunohistochemical staining was performed to detect the senescent cells. The decalcified sections

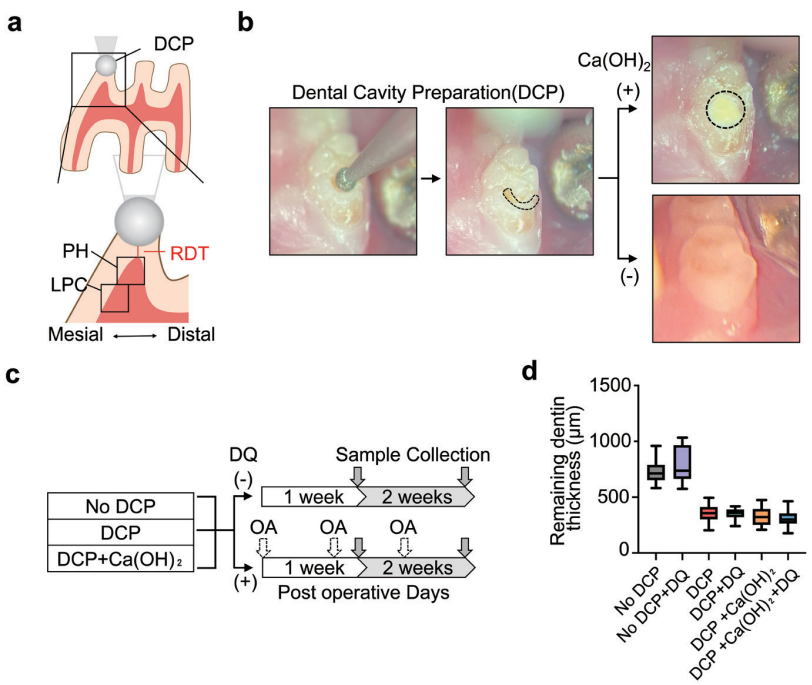

Fig. 1 Non-pulp-exposed DCP model.
(a) Schematic illustration of DCP model. PH: pulp horn, LPC: lateral side of the pulp chamber, RDT: remaining dentin thickness. (b) Macroscopic images of DCP and pulp capping utilizing calcium hydroxide ($Ca(OH)_2$). All samples were temporarily sealed by dental adhesive resin cement. (c) Workflow of animal experiments with or without dasatinib+quercetin (DQ) administration. OA: oral administration. (d) Quantitative analysis of remaining dentin thickness without repaired dentins to verify the original distance from the bottom of cavities to pulps. Data are presented as the mean with standard deviation (S.D.) (n=4).

obtained from the above procedure were blocked and permeabilized with 5% goat serum and 0.3% Triton X-100 in phosphate-buffered saline (PBS). The sections were then incubated with the following primary antibodies: p21 polyclonal antibody conjugated with Alexa Fluor 555 (bs-10129R-A555, BIOSS Antibodies, Boston, MA, USA; 1:100) and CDKN2A/p16-INK4a polyclonal antibody conjugated with Alexa Fluor 488 (bs-23881R-A488, BIOSS Antibodies; 1:100). Sections were washed in PBS and treated with 4',6-diamidino-2-phenylindole (DAPI Fluoromount-G, Southern Biotech, Birmingham, AL, USA). All fluorescence images were acquired using a laser confocal microscope (ZEISS LSM700; Carl Zeiss, Jena, Germany). Senescent cells in the region of interest in the PH and LPC were quantified using ImageJ (version: 2.1.0, U.S. National Institutes of Health) as the ratio of the area of positive staining for p21 or p16 to the area of DAPI according to the following equation: [(area of positive antibody staining/area of DAPI)×100]. The average ratio of the four images was used as an individual sample (Figs. 2b and 3b).

Preparation and administration of the senolytics dasatinib and quercetin

To prepare the dasatinib (Cayman Chemical, Ann Arbor, MI, USA) and quercetin (SCB Santa Cruz Biotechnology, Dallas, TX, USA) solutions, both reagents were dissolved in PEG-200 with MilliQ. After weighing each rat in the group, the rats were orally administered 6.67 mg/kg dasatinib and 66.7 mg/kg quercetin on the day of preparing DCP and then once every 5 days for 2 weeks. Senescent cells in the region of interest in the PH or LPC were quantified using ImageJ (version: 2.1.0, U.S. National Institutes of Health) at the ratio of the area of positive staining for p21 and p16 against the area without DQ. The average ratio of the four images was used as an individual sample (Figs. 4b and 5b).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance, followed by the Tukey-Kramer test to determine significance for multiple comparisons. Student's *t*-test was performed for two groups comparisons. GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

RESULTS

Establishment of a non-pulp-exposed DCP model

To confirm the stability of the DCP, the thickness of the remaining dentin was evaluated histologically using H-E staining (Fig. 1d). The original distance from the bottom of the cavities to the pulp was estimated without considering the thickness of reparative dentin (Figs. 1a and d). All samples had a thickness of more than 150 μm, confirming the establishment of the nonexposed dentin DCP model (Fig. 1d).

Senescent cells in the dental pulp after DCP with or without indirect pulp capping with $Ca(OH)_2$

To elucidate the presence of senescent cells after DCP, we evaluated p21 and p16 expression in the PH and LPC regions one and two weeks after the operation using immunohistochemical staining (Figs. 2 and 3). In the PH region, p21- and p16-positive cells increased in the DCP and DCP+$Ca(OH)_2$ groups after 1 week. The DCP group maintained p21- and p16-positive cells at two weeks, but there was a decrease in the DCP+Ca $(OH)_2$ group (Fig. 2b). In the LPC region, a few p21-positive cells were observed in each group at 1 week (Fig. 2b), whereas the DCP group showed an increase in p16-positive cells at 1 week (Fig. 3b). At 2 weeks, the area ratio of p21- and p16-positive cells increased or was maintained in the DCP group (Figs. 2b and 3b). These results indicate that DCP treatment increased the number of p21 and p16 senescent cells. In contrast, this increase was attenuated with time in the DCP+$Ca(OH)_2$ group.

Administration of senolytics after DCP with or without indirect pulp capping with $Ca(OH)_2$

We examined whether senolytics could remove senescent cells after DCP using immunohistological analysis (Figs. 4a and 5a). The DQ-administered groups showed a decrease in the number of p21- and p16-positive cells up to 2 weeks (Figs. 4b and 5b). In the DCP+$Ca(OH)_2$ group, the DQ-administered groups showed a decrease in the area ratio of p21- and p16-positive cells after 1 week (Figs. 4a and 5a). These results indicate that the oral administration of DQ can decrease the number of p21- and p16-positive senescent cells induced by DCP.

Histomorphometric analysis of the reparative dentin formation

To evaluate the relationship between DCP, DQ, $Ca(OH)_2$, and reparative dentin formation, we investigated PH and LPC using H-E staining (Fig. 6). Reparative dentin is observed discontinuous of dentinal tubles and pale colored unlike the original dentin in H-E. We could not find marked reparative dentin formation in the PH or LPC groups from the non DCP, non DCP+DQ, and DCP groups at 1 week. In contrast, DCP with DQ administration and $Ca(OH)_2$ showed remarkably increased dentin formation in PH at 1 week. However, there was no obvious increase from 1 to 2 weeks in either the DCP or DCP+$Ca(OH)_2$ groups after DQ administration. Additionally, there was a negligible difference in DCP+$Ca(OH)_2$ with and without DQ groups. These results indicated that DQ administration restored the early stages of dentin formation aggravated by DCP.

DISCUSSION

In this study, we observed an increase in the number of senescent cells presenting conventional senescence markers (p21 and p16) in the pulp. Additionally, the oral administration of DQ reduced the number of senescent cells and remarkably restored the volume of reparative dentin formation, similar to indirect pulp capping.

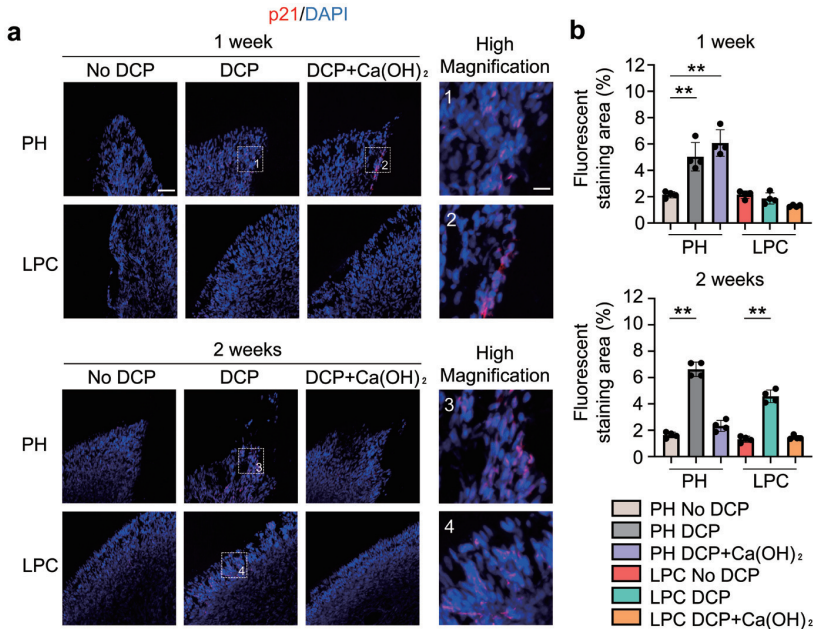

Fig. 2 Localization of p21-positive cells in the dental pulp.
(a) Immunofluorescence and high-magnification images of pulps stained with senescence marker (p21) and 4',6-diamidino-2-phenylindole (DAPI). Scale bars: low magnification, 40 μm; high magnification, 10 μm. DCP: dental cavity preparation, PH: pulp horn, LPC: lateral side of the pulp chamber. (b) Quantitative analysis of p21-positive area at the region of the PH and LPC. Data are presented as the mean with S.D. (n=4). One-way ANOVA with Tukey-Kramer; **p<0.001

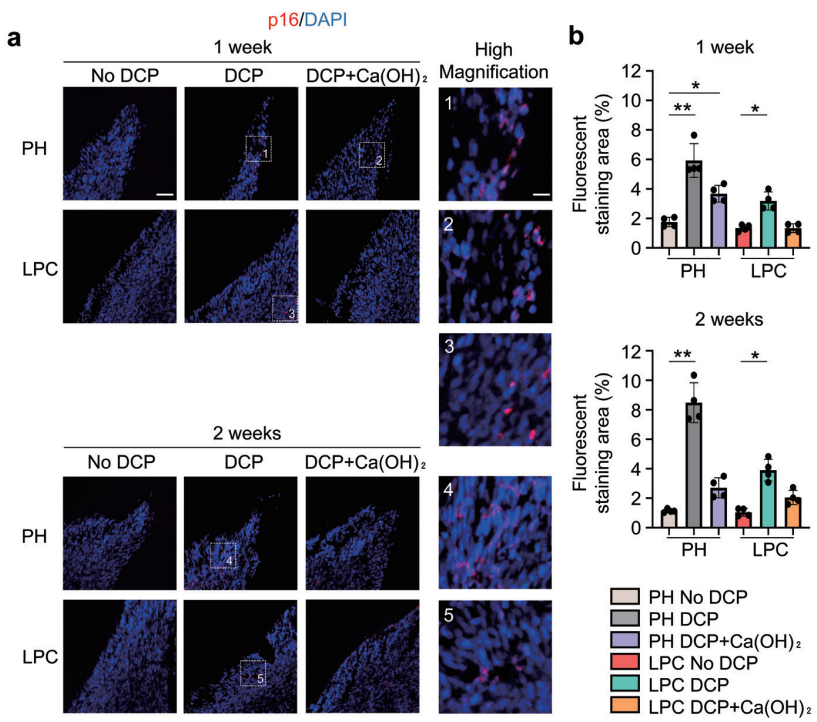

Fig. 3 Localization of p16-positive cells in the dental pulp.
(a) Immunofluorescence and high-magnification images of pulp stained with senescence marker (p16) and DAPI. DCP: dental cavity preparation, PH: pulp horn, LPC: lateral side of the pulp chamber. Scale bars: low magnification, 40 μm; high magnification, 10 μm. (b) Quantitative analysis of p16-positive area at the region of the PH and LPC. Data are presented as the mean with S.D. (n=4). One-way ANOVA with Tukey-Kramer; *p<0.01, **p<0.001

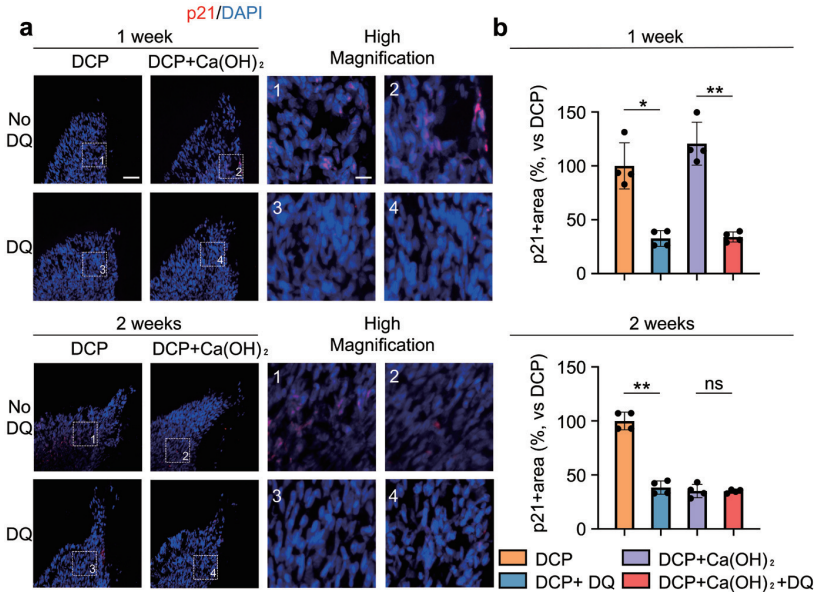

Fig. 4 p21-positive cells in the dental pulp with or without DQ administration.
(a) Immunofluorescence and high-magnification images of pulp stained with senescence marker (p21) and DAPI. DCP: dental cavity preparation. Scale bars: low magnification, 40 μm; high magnification, 10 μm. (b) Quantitative analysis of p21-positive area at the region of the pulp horn with or without DQ administration. Data are presented as the mean with S.D. (n=4), Student's t-test; *p<0.01, **p<0.001.

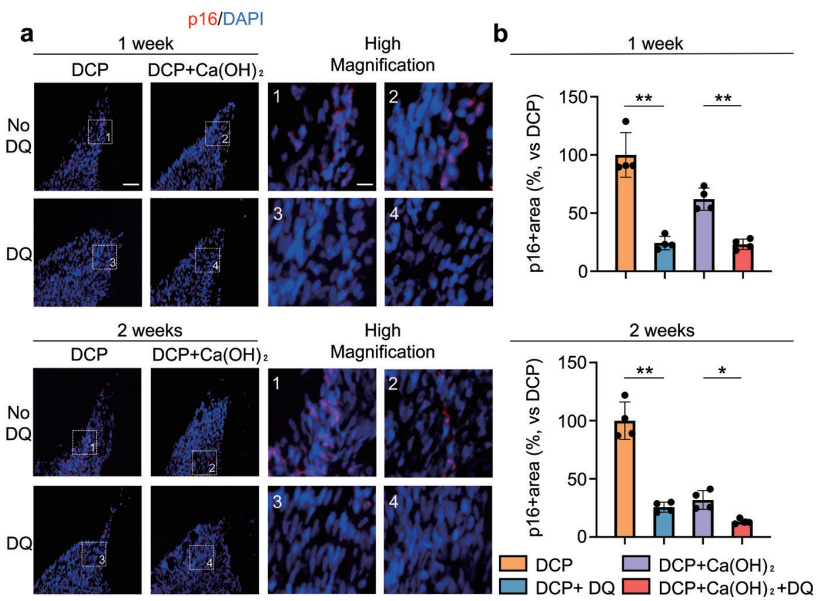

Fig. 5 p16-positive cells in the dental pulp with or without DQ administration.
(a) Immunofluorescence and high-magnification images of pulp stained with senescence marker (p16) and DAPI. DCP: dental cavity preparation. Scale bars: low magnification, 40 μm; high magnification, 10 μm. (b) Quantitative analysis of p16-positive area at the region of the pulp horn with or without DQ administration. Data are presented as the mean with S.D. (n=4), Student's t-test; *p<0.01, **p<0.001

To date, various animal DCP models have been developed to evaluate reparative dentin formation[29,30]. These DCP models are roughly classified as pulp-exposed models[31,32] and non-pulp-exposed models[33,34]. Pulp exposure increases the risk of inducing pulp necrosis[35,36], which potentially veils the cellular behavior of senescent cells for reparative dentin formation. Thus, in the current study, we selected and prepared a non-pulp-exposed DCP model (Fig. 1a), which was suitable for a clearer analysis of the cellular dynamics of senescent cells after

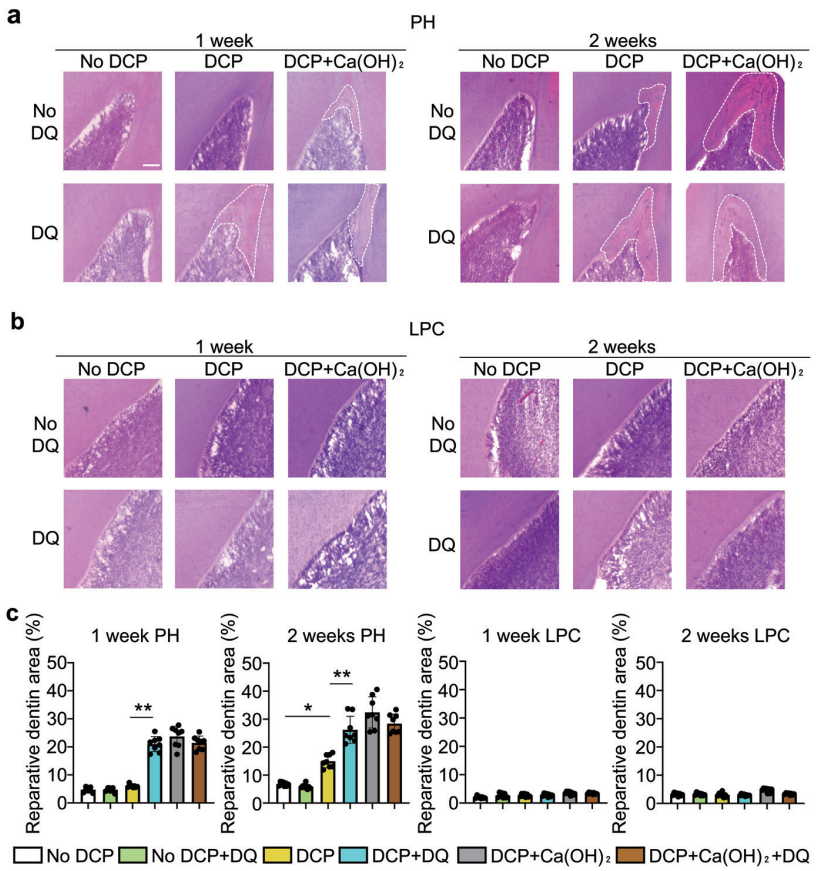

Fig. 6 Histological evaluation of reparative dentin formation.
(a, b) Representative hematoxylin-eosin staining images of the pulp horn (PH) and lateral side of the pulp chamber (LPC) region after operation. DCP: dental cavity preparation, dotted line: reparative dentin (RD). Scale bars, 40 μm. (c) Histomorphometric analysis of RD area. Data are presented as the mean with S.D. (n=8), One-way ANOVA analysis with Tukey-Kramer; *p<0.01, **p<0.001

DCP (Fig. 1a).

The p53/p21$^{WAF1/CIP1}$ and p16^{INK4A}/pRB tumor suppressor pathways are considered to be central signaling pathways associated with cellular senescence[37-39]. Thus, to confirm the presence and localization of senescent cells, we histologically analyzed the expression of p21 and p16, the core proteins of each signaling pathway, as senescence markers. Both p21- and p16-positive senescent cells were found in the pulp one week after DCP. Senescent cells were particularly localized in the PH area and gradually expanded to the LPC area two weeks after DCP. These results indicate that some stimuli (stress) caused by DCP may induce cellular senescence and propagate directly or indirectly from PH to LPC in pulp tissues.

DCP potentially causes a variety of stresses, including heat and mechanical stress[40,41]. Endogenous and exogenous stresses in the body are known to provoke cellular senescence, the so-called stress-induced premature senescence of cells representing p21 and p16 expression[42], in various organs, leading to diseases[13,42]. Although we could not verify the detailed stressors under the DCP, in view of p21 and p16 expression in the pulp, DCP seems to induce some stress and cause local cellular senescence.

Previous study found that DQ can eliminate senescent cells[23]. By modifying the dose and frequency of DQ administration, we have established the method that can eliminate senescent cells in the cranial bone defects of rats[43,44]. In this study, we found the dose and frequency of DQ administration that can eliminate senescent cells under the DCP with reference to past conditions.

The formation of reparative dentin is initiated by undifferentiated pulp mesenchymal stem cells that differentiate into odontoblasts[45,46]. Proliferation of pulp stem cells and mineralization caused by odontoblasts closely affect reparative dentin formation[47]. In contrast, senescent pulp cells are known to have reduced proliferation and differentiation capacity[48,49]. In this study, the formation of reparative dentin was observed around the PH, where senescent cells are abundant before DQ administration. However, these cells are not always observed near the dentin surface, suggesting that the origin of senescent cells is not always odontoblasts but also other types of cells. These senescent cells may

directly or indirectly hamper the proliferation and odontoblastic differentiation of the pulp stem cells.

In our study, DCP with $Ca(OH)_2$ formed reparative dentin after 1 week, despite the induction of senescent cells in the pulp. Although there is no clear evidence to support these controversial findings, $Ca(OH)_2$ promotes the formation of reparative dentin by increasing recruitment, migration, proliferation, and mineralization[50]. Furthermore, $Ca(OH)_2$ increases pH[51], and environmental changes may alter the function of various proteins around odontoblasts. These effects might restore odontoblasts to form dentin or hamper the deleterious functions of some SASPs secreted by senescent cells.

Mastication, occlusion, and bruxism have been reported to cause pulp cell senescence[52]. Additionally, there is consensus that these diseases, aging, and DCP occasionally trigger reparative dentin formation[53]. Considering these findings, not all senescent cells exacerbate dentin formation. Indeed, our data also showed that DCP induced reparative dentin formation from 2 weeks, despite the presence of senescent cells. More recently, much research has distinguished senescent cells by type, stage, stress factor, and origin[54-58]. Consequently, further detailed studies are essential to understand the function of stress-induced senescent cells, possibly induced by DCP. However, our results provide valuable insights into the function of senescent cells in the pulp to develop a new approach in restorative dentistry.

CONCLUSION

In the present study, we demonstrated that stimulation by cavity formation induces senescent cells in the dental pulp. Additionally, the oral administration of senolytics (dasatinib and quercetin) significantly accelerated reparative dentin formation under DCP (Fig. 7). In the present study, we could not verify the type of cells that underwent cellular senescence in the pulp and hampered the reparative dentin formation. However, given the localization of p21- and p16-positive senescent cells in the pulp and the effect of senolytics on restoring dentin formation, the removal of senescent cells in the pulp may improve the deleterious environment that aggravates the proliferative and differentiation ability of stem cells. Using senolytics is a promising approach for inducing reparative dentin formation after DCP.

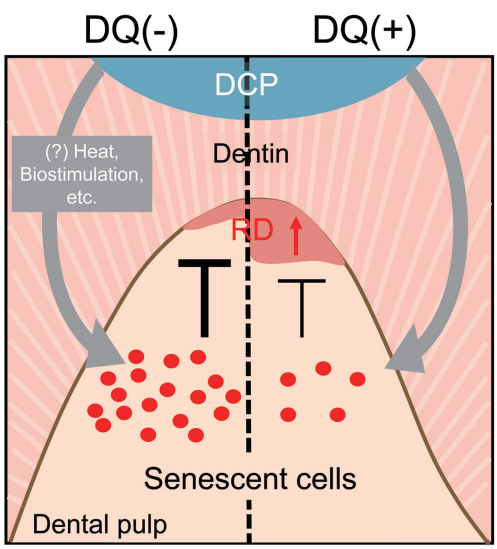

Fig. 7 Schematic illustration of possible relationship between cavity preparation, senescent cells, and reparative dentin.

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