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Novel strategy for dental caries by physiologic dentin regeneration with CPNE7 peptide

CPNE7 펩타이드의 생리적 상아질 형성을 통한 치아 우식증의 새로운 치료 방법

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ABSTRACT

Novel strategy for dental caries by physiologic dentin regeneration with CPNE7 peptide

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CPNE7-derived functional peptide (CPNE7-DP) has been introduced as bioactive therapeutics for dentin diseases. CPNE7-DP regenerates tubular dentin on the pulpal side and occlude dentinal tubules. CPNE7-DP was capable to treat dentin hypersensitivity typically associated with dentinal wear at the neck of the tooth. However, the role of CPNE7-DP in another common dentin disease, dental caries, remains uninvestigated. In this study, the potential application of CPNE7-DP in dentin caries was evaluated using an experimental dentin caries model in rats.

The stability of CPNE7-DP in caries-like environments including pathologic bacteria of caries or low pH was tested. I established a nutrition-time/hyposalivation-based dental caries rat model by inoculating caries-inducing bacteria and diet for sufficient time. Glycopyrrolate has been treated to induce reversible hyposalivation for accelerating caries progression. Then the tubular dentin regeneration was investigated with histologic methods. Also, modulation of inflammation or autophagy by CPNE7-DP was investigated with marker gene expression in human dental pulp cells (hDPCs) and immunohistochemistry.

CPNE7-DP was stable with caries-inducing bacteria and low pH. Establishment of dentin caries was confirmed with radiographic and histologic evaluation. CPNE7-DP regenerated a substantial amount of tubular tertiary dentin and alleviated the pulp inflammation of dentin caries. Under inflammatory conditions, CPNE7-DP reduced the expression of inflammatory cytokines. These phenomena could be the consequence of the modulation of autophagy by CPNE7-DP, which reactivates inflamed odontoblasts.

Overall, CPNE7-DP, which arrests caries through physiological dentin regeneration, might help overcoming the limitations of current restorative caries treatments.

Keywords: Regenerative medicine, Minimal invasive dentistry, Caries treatment, Odontoblast(s), Bioactive materials, Pulpitis

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I. INTRODUCTION

1. Dentin

Dentin is mineralized connective tissue with organic matrix. It forms majority of teeth beneath enamel. The composition of mature dentin is approximately 70% inorganic materials, 20% organic materials and 10% of water (Marshall, 1993). After eruption, the external stress such as attrition, trauma, or dental caries reactivate odontoblasts to secrete tertiary dentin (Klinge, 2001). Tertiary dentin is divided into reactionary or reparative dentin. If the odontoblast layer is intact, reactionary dentin showing dentinal tubules is formed (Smith et al., 1995). Contrarily, reparative dentin is formed when the odontoblast layer is replaced due to severe damage (Cox et al., 1992).

Unlike the reparative dentin, when the reactionary dentin is regenerated at caries lesions, the bacteria can no longer infiltrate to the calciotraumatic zone, thereby protecting pulp from the inflammation. In order to arrest dentin caries effectively and maintain the defensive role of dentin even with the selective excavation, it is pivotal to have the “physiological dentin” as a tertiary dentin.

2. Copine-7 (CPNE-7) and Copine-7-derived peptide (CPNE7-DP)

The copine family are ubiquitous calcium dependent phospholipid-binding proteins. They are highly conserved through evolutions from *Arabidopsis* to *Homo sapiens* (Tomsig et al., 2003). The novel protein Copine 7 (CPNE7, 633 aa) was identified in pre-ameloblast conditioned medium (PA-CM) as a candidate signaling molecule regulating odontoblast differentiation and periodontium formation (J. H. Lee et al., 2011).

CPNE7-DP is CPNE7 protein-derived synthetic peptide which has similar function like CPNE7 and cell penetrating effect. CPNE7-DP has been introduced and examined as a potential bioactive for dentinal diseases. CPNE7-DP displayed capacity to induce odontoblast differentiation and form physiological dentin, suggesting its potential to cure dentin hypersensitivity or dentinal defects (Y. S. Lee et al., 2020; S. H. Park et al., 2019).

3. Prevalence and social burden of dental caries

Dental caries, which affect approximately 3.5 billion people worldwide, is the most common chronic disease (Dye, 2017; Kassebaum et al., 2015; World Health Oraganization, 2017). Dental caries progression eventually causes an edentulous state with chewing disability, which severely affects

quality of life (Bortoluzzi et al., 2012). Therefore, treatment for dental caries is essential to improve the life quality. Still, many are unable to receive dental care due to such reasons as dental phobia (Appukuttan et al., 2015; Enkling et al., 2006; Pohjola et al., 2016) or social inequality (Vujicic et al., 2016; Williams et al., 2021).

4. Etiology of dental caries

The main etiologic factors for dental caries are bacteria, time, tooth surface, fermentable carbohydrates. Dental caries is infectious disease caused by imbalance of the native oral microbiome (Caufield et al., 2005). The bacteria, such as *Streptococcus mutans*, colonizes the tooth surface and secrete acidic byproducts. The sucrose, representative fermentable carbohydrates, is hydrolyzed by *Streptococcus mutans*, producing lactic acid to decalcify the tooth surface. *S. mutans* is considered as primary bacteria to form the dental plaque (Köhler et al., 1981). At early stage of dental caries such as development, gram-positive bacteria start the infection. As the carious lesion progress deeper into the pulp-dentin, the number of gram-negative increases (Horst et al., 2009).Eventually the tooth surface become soft and brittle leading to destruction of tooth structure (Kidd et al., 1996; Çehreli et al., 2003). Due to its infectious etiology, dental caries differs from other destructive dental diseases like cervical abrasion.

5. Dental caries model

Various *in vitro* and *in vivo* technique have been used for dental caries, but many of them are focusing on biofilm formation or bacterial inhibition rather than demineralization. (Li et al., 2014a, 2014b) Most of animal model for demineralization are limited on enamel caries model.

For dental caries animal model, non-human primates, large animals and murine models have been used previously. The teeth size of large animals including beagle dogs are similar to human. This feature makes the clinical procedure to be same as in humans.(Aubeux et al., 2021) Also dogs have biochemical and physiologic characteristics close to human. (Nakashima et al., 2019) For the last, murine model has been widely used due to its cost and easiness to handle. The immune system of murine is quite similar except the blood cell proportion of neutrophil and lymphocytes, Toll-like receptors, defensins, cytokines and cytokine receptors. (Mestas & Hughes, 2004) Rodent caries model is like human in genetic and hard tissue composition aspects.

6. Caries treatment

Generally perceived, the ultimate goal of caries treatment is to alleviate pulp inflammation (Zero et al., 2011). To achieve the objective, two different treatment approaches are introduced: non-selective and selective excavation.

The non-selective excavation refers to the total removal of caries-infected dentin (Schwendicke et al., 2016). In this way, cariogenic bacteria are no longer residing at the teeth. The selective excavation is the partial removal of infected dentin followed by the dentinal sealing with restorative materials such as resin. Although there should be some remnants of bacteria at the dentinal tubule, the appropriate placement of restoration can seal the dentinal tubule surface to remove bacteria residing in the tubule by forming the anaerobic environment. However, as the restorative materials are not permanently durable, the elimination of bacteria is only temporary. In other words, selective excavation is merely a stopgap for the dental caries (Ricucci et al., 2020a). Resolving limitations of non-selective and selective excavations, biological dentin sealing, the regeneration of the tertiary dentin with occluded upper end of dentinal tubule surface, arises as an optimal and practical agent for dentin caries (S. H. Park et al., 2019).

7. Autophagy

Autophagy is an intracellular recycling process which degrades dysfunctional cellular components in the lysosomes (Mizushima, 2007). Although autophagy indicates macroautophagy, there are other types such as microautophagy and chaperon-mediated autophagy (Glick et al., 2010). Autophagy has been revealed to participate in various physiological and pathological process. The significance of this cellular metabolism has been

dated in anti-aging, eliminating microbes, adapting starvation and inflammation (Mizushima, 2007). Moreover, previous findings imply autophagy is crucial in pulpitis to protect cells from pyroptosis (Kitagawa et al., 2007). This finding was further confirmed by the high level of autophagy, especially at the odontoblast layer, in irreversible pulpitis (Qi et al., 2019).

8. Rationale and outline of the thesis experiments

A key purpose of this thesis is to investigate the role of CPNE7-DP on dental caries. To examine this goal, stability of CPNE7-DP in caries-like environment was investigated. Then, I developed *in vivo* dentin caries models, which has been very rare despite the generality of the dental caries. I also evaluated the capacity of CPNE7-DP forming physiological dentin at the caries lesions to explore the possibility of CPNE7-DP as an ideal but practical therapeutic agent for arresting dental caries. To figure out how CPNE7-DP alleviated pulpitis in dentin caries, expression level of inflammatory cytokine was evaluated with CPNE7-DP treatment in hDPC pulpitis model. Also, the effect of CPNE7-DP on cell survivability, mineralization capacity and wound healing capacity of hDPC was investigated.

II. MATERIALS AND METHODS

1. Dentinal tubule penetration in dentin caries lesion

The experimental procedures for this study were approved by the Institutional Review Board of Seoul National University Dental Hospital (S-D20140007). All experimental protocols were performed in accordance with the relevant guidelines and regulations. Third molars were collected from patients who signed an informed consent form at the Seoul National University Dental Hospital (Seoul, South Korea). Rhodamine-labeled CPNE7-DP (10 μg/tooth) was topically applied to the dentin caries lesions of extracted human molars (n=10) for 1 min. The teeth were then fixed with 4% paraformaldehyde (PFA; T&I, BFA-9020, Kyunggi-Do, Korea) and sectioned sagittal at 0.1 mm thickness using a digital low-speed diamond saw (MTI, SYJ-160, CA, and USA). The sectioned samples were visualized using confocal laser scanning microscopy (Carl Zeiss, LSM 800, Oberkochen, Germany).

2. Matrix-assisted laser desorption/ionization imaging coupled to time-of-flight (MALDI-TOF)

CPNE7-DP (100 μg) was exposed to different conditions including various pHs (7.4, 6.0, 5.3) and *S. mutans* for 30 mins. The samples were washed with 1 mL of ethanol at −20°C and incubated with thoroughly vortex-mixed solution overnight. Then, the samples were centrifuged for 15 min at 16,100 × *g* at 4°C, and the supernatant was aspirated. The remaining pellet was resuspended and incubated with 30 μL of 0.1 M Triethanolamine containing β-mercaptoethanol buffer (TEAM buffer) for 2 h at 37°C. Subsequently, 2 μL of ice-cold A tri-buffer system (APT buffer) was added to 0.8 μL of the samples, which were dispensed into the cleaned anchor chip target plate. The recrystallization buffer was treated after complete air drying of the anchor spots, and the samples were analyzed using MALDI-TOF mass spectrometry (MALDI-TOF/MS). The analysis was performed in three independent experiments.

3. Circular Dichroism (CD)

The secondary structure of CPNE7-DP was studied at different pH values (pH 5.4, 6.0 and 7.4) after 1 hr of incubation. The peptide solutions were prepared in 1mg/mL concentration. CD spectra were collected on Circular

Dichroism Detector (Chirascan plus, Applied Photophysics, UK) in a rectangular quartz cell with a 0.2mm path length. Wavelength was scanned from 180 to 280 nm. All experiments were performed in three independent experiments. The data was analyzed using CDNN secondary structure analysis software.

4. Nutrition-time based dental caries model in Sprague-Dawley (SD) rats

All experiments using SD rats were conducted according to the guidelines provided by the Institutional Animal Care and Use Committee of Seoul National University (SNU-181127-3) and ARRIVE 2.0 guidelines. The normal microflora in the oral cavity of SPF SD rats (3-week-old males) were suppressed to facilitate opportunistic infection by caries-inducing bacteria. They were treated with penicillin G (10,000 U/mL of water), streptomycin (10,000 μg/mL), and fungizone (amphotericin, 25 μg/mL) for three days. All the rats were inoculated with a bacterial suspension (*Streptococcus mutans* 2×10^9 CFU/100 μL, *S. oralis* 4×10^8 CFU/100 μL, *S. mitis* 4×10^8 CFU/100 μL, and *S. sanguinis* 2×10^8 CFU/100 μL) mixed with carboxymethyl cellulose. The bacterial suspension was treated for five consecutive days. As the inoculation began, the rats were fed a caries-inducing diet (Diet #2000)

comprising 56% sucrose. After 1.5 months, the rats were reinoculated with an *S. mutans* (2×10^9 CFU/100 μL) suspension. To accelerate the caries progression, hyposalivation was induced with glycopyrrolate, administered at incremental doses (0.75 mg/kg p.o. q.d. for 5 days, then 1.0 mg/kg p.o. q.d. for 7 days). After following the schedule for experimental caries, the rats were anesthetized with isoflurane (FORANE-Baxter international Inc., NDC 66794-017-25, IL, USA) 4-5% for induction and 1-2% for maintenance. The oxygen flow rate was 300-600 ml/min. The oral cavity was opened with a mouth retractor. PBS (caries only group) or 3 μg CPNE7-DP (1 μg/μL, experimental group) was topically applicated on all maxillary molars for 1 min. The animals were fed with ordinary diet after the treatment and sacrificed after 3 weeks post-operation for histological analysis.

5. Micro-CT scanning

The heads of mice were fixed in 4% PFA in PBS for 24 h at 4 ℃. After fixation, the samples were scanned with a micro-CT scanner (Bruker, Skyscan 1172, MA, USA) with a 7-μm voxel size. Data were analyzed using micro-CT imaging software (PerkinElmer, AccuCT micro CT Analysis CLS146216, MA, USA).

6. Tissue preparation and histology (hematoxylin and eosin [H&E] and bacterial staining)

Tissue preparation and H&E staining were performed as previously described (Y. S. Lee et al., 2020). The samples were fixed in 4% PFA (T&I, BFA-9020, Kyunggi-Do, Korea) at 4°C overnight, decalcified with 10% ethylenediaminetetraacetic acid (EDTA; pH 7.4; Georgiachem, ED2041, Florida, USA), and embedded in paraffin. The embedded tissue was serially sectioned into 4-μm slices, mounted on silanized slides, deparaffinized with xylene (DUKSAN Chemicals, UN1307, Kyunggi-Do, Korea), then rehydrated in ethanol (DUKSAN Chemicals, UN1170, Kyunggi-Do, Korea). For H&E staining, tissue sections were incubated in hematoxylin (Vector Labs, H-3401, CA, USA) for 4 min and eosin (T&I, BEY-9005, Kyunggi-Do, Korea) for 1 min at 25°C. For bacterial staining, a bacteria detection kit (Newcomer supply, Part#9123A, WI, USA) was used per the manufacturer's recommendations.

7. Cell culture

The experimental procedures for this study were approved by the Institutional Review Board of Seoul National University Dental Hospital (S-D20140007). All experimental protocols were performed in accordance with

the relevant guidelines and regulations. Third molars were collected from patients who signed an informed consent form at the Seoul National University Dental Hospital (Seoul, South Korea). The pulp was isolated from the extracted teeth as previously described (H. W. Choung et al., 2016). Human dental pulp cells (hDPCs) were cultured for osteogenic differentiation as previously described (Y. H. Park et al., 2021).

8. Real-time PCR analysis

CPNE7-DP (10μg/mL) and rCPNE7 (1μg/mL) were treated on hDPCs (n=3) to evaluate CPNE7 expression. Also, Lipopolysaccharide (1μg/mL, LPS) and CPNE7-DP (10μg/mL) were treated simultaneously or sequentially on hDPCs (n=3). Then the expression of TNF-α, IL-1α, IL-1β, IL-6, CXCL10, iNOS, and COX2 was evaluated with real-time PCR. Real-time PCR Tri Reagent (Invitrogen, 15596018, TRIzol Reagent, MA, USA) was used for total RNA extraction from the cells. Reverse transcription was performed using reverse transcriptase (RTase; Invitrogen, 18080093, MA, USA) and oligo primers (Invitrogen, AM5730G, Massachusetts, USA) were provided to the total RNA. For amplification, 1 μL of a reverse transcription (RT) product, gene-specific primer, and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, 1725125, CA, USA) were incubated for several cycles using a 96-well RT polymerase chain reaction (PCR) machine (Applied Biosystems,

4376600, CA, USA). The PCR conditions were 40 cycles of 95 ℃ for 1 min, 94℃ for 15 s, and 60℃ for 34 s. The PCR product level of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was further calculated into relative changes using the comparative threshold cycle (C_T) method. All reactions were analyzed in triplicate. The primer sequences are listed in Table 1.

9. Western blot analysis

Western blot analysis was performed as previously described (Y. M. Seo et al., 2017). Antibodies against rCPNE7 and DSP were produced as previously described (J. H. Lee et al., 2011). Anti-LC3 B polyclonal antibody (Abcam, ab48394, Cambridge, UK) and anti-GAPDH monoclonal antibody (GA1R; Thermo Fisher Scientific, MA5-15738, MA, USA) were used. All results were confirmed in triplicate with semi-quantitative analysis using the ImageJ software (National Institutes of Health, MD, USA).

10. Immunohistochemistry

Sections were incubated with rabbit polyclonal LC3B antibody (1:200) or ED1 (1:100; Abcam, ab31630, Cambridge, UK) in 2% BSA/PBS (pH 7.4) at 4°C overnight. Negative controls were incubated with 2% BSA/PBS alone.

Biotin-labeled goat anti-rabbit immunoglobulin G (IgG; 1:200; Vector Labs, BP-9100, CA, USA) was used as the secondary antibody. Incubated sections were washed and treated with avidin-biotin-peroxidase complex (Vector Labs, PK-6100, CA, USA). Finally, 3% H_2O_2 (JUNSEI, 7722084-1, Changshu, China) and the diaminobenzidine kit (Vector Labs, SK-4100, CA, USA) were used to detect the signals. Hematoxylin was used to counterstain the nuclei. ED1 positive cells were counted at two or three different random part of coronal pulp depending on the pulp size and interpreted by three blinded investigators.

11. Cell proliferation assay

Primary hDPCs (passage 2-4) were seeded at a density of 5×10^3 cells/well in a 96-well plate and incubated at 37°C with CO_2. For the cell proliferation assay, each well was treated with 150 μL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide solution, Medifab) for 2 h at 25℃ on days 0, 1, 3, and 5. The cells were then lysed with DMSO (Duksan Chemicals, CAS 67-68-5, Kyunggi-Do, Korea), and absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Synergy HTX, VT, USA). The experiments were done in sextuplicate.

12. Wound healing assay

Primary hDPCs (passage 2-4) were seeded into 6-well culture dishes at a density of 1×10^5 cells/well then grown to 100% of monolayer confluency. Gentle scratching across the culture plate with sterilized 200 μL tip was applied to every well. Then the cells were incubated in cell medium with LPS or LPS/CPNE7-DP for 24 hrs. Cell migration rates were analyzed through the distribution of wound filling cells with ImageJ software (National Institutes of Health, MD, USA). All the experiments were performed in triplicates.

13. Alizarin red staining

Primary hDPCs (passage 2-4) were seeded into 6-well culture dishes at a density of 1×10^5 cells/well. The cells were then cultured with LPS (10μg/mL)-, LPS/CPNE7-DP (both 10μg/mL)-, and CPNE7-DP (10μg/mL)-treated odontogenic differentiation medium for 7, 14, and 21 days. For fixation, the cells were incubated with 4% PFA overnight at 4°C and stained with 40 mM alizarin red S (Sigma-Aldrich, A5533, MA, USA) calibrated for pH 4.2 at room temperature for 30 min. The mineralized matrix was quantified by incubation with 0.5 mL of 5% sodium dodecyl sulfate (Amresco, 0227, OH, USA) in 0.5 N HCl and shaking for 30 min. The absorbance was then measured at 405 nm. All experiments were performed in triplicates.

14. Statistical analysis

Data were analyzed using GraphPad Prism 8.0.2 (GraphPad Holdings, LLC, CA, USA). All values were expressed as means ± SDs obtained from at least three independent experiments. Mann-Whitney U test (Wilcoxon rank-sum test) was used to compare the two distinct groups. For the comparison between groups of three or more, one-way ANOVA was used. Differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Table 1. Lists of primer sequence for RT-PCR

Name of the gene	RT-PCR primer sequence
CPNE7	F : 5'-CGGGACCCATTGACCAAGTC-3' R : 5'-CATACACCTCAAACCGTAGCTTC-3'
TNF-α	F : 5'-CCTGGTATGAGCCCATCTATCTG-3' R : 5'-GCAATGATCCCAAAGTAGACCTG-3'
IL-1α	F : 5'-TGTATGTGACTGCCCAAGATGAAG-3' R : 5'-AGAGGAGGTTGGTCTCACTACC-3'
IL-1β	F : 5'-CCAGGGACAGGATATGGAGCA-3' R : 5'-TTCAACACGCAGGACAGGTACAG-3'
IL-6	F : 5'-CGAAAGTCAACTCCATCTGCC-3' R : 5'-GGCAACTGGCTGGAAGTCTCT-3'
CXCL10	F : 5'-TGCCATTCTGATTTGCTGCC-3' R : 5'-TGCAGGTACAGCGTACAGTT-3'
iNOS	F : 5'-CAGCGGGATGACTTTCCAA-3' R : 5'-AGGCAAGATTTGGACCTGCA-3'
COX2	F : 5'-TTCTCCTTGAAAGGACTTATGGGTAA-3' R : 5'-AGAACTTGCATTGATGGTGACTGTTT-3'
BECLIN 1	F : 5'-CAAGATCCTGGACCGTGTACA-3' R : 5'-TGGCACTTTCTGTGGACATCA-3'
ATG3	F : 5'-TCACAACACAGGTATTACAGG-3' R : 5'-TCACCGCCAGCATCAG-3'
ATG5	F : 5'-GGGAAGCAGAACCATACTATTTG-3' R : 5'-AAATGTACTGTGATGTTCCAAGG-3'
ATG7	F : 5'-AGGAGATTCAACCAGAGACC-3' R : 5'-GCACAAGCCCAAGAGAGG-3'
GAPDH	F : 5'-GGGAAGCTTGTCATCAATGG-3' R : 5'-CATCGCCCCACTTGATTTTG-3'

III. RESULTS

1. CPNE7-DP was stable in various stress conditions of dental caries

To clarify the availability of CPNE7-DP on caries lesions, the stability of CPNE7-DP was first examined under stress conditions of caries lesions, including modifications to the pH and cariogenic bacteria. I first confirmed the stability of CPNE7-DP by exposing the peptide to *S. mutans,* the pathogenic bacteria of dental caries (Figure 1A). Furthermore, CPNE7-DP was also stable in various neutral to acidic environment, in which dentin caries were likely to progress due to the acids secreted from bacteria (Figure 1B). The peptide structure was not modified under these conditions. At pH where the peptide chain may not be cleaved, its natural spatial structure could be altered, leading to loss of biological activity. In the neutral to acidic pH, the secondary structure of CPNE7-DP was maintained which further confirmed its stability (Figure 1C). The penetrating ability of CPNE7-DP through dentinal tubules in caries lesions was confirmed by confocal microscopy (Figure 1D). CPNE7-DP was detected throughout the length of the dentinal tubules and pulp. These results suggest CPNE7-DP is stable in caries lesion and possibly penetrates well into pulp.

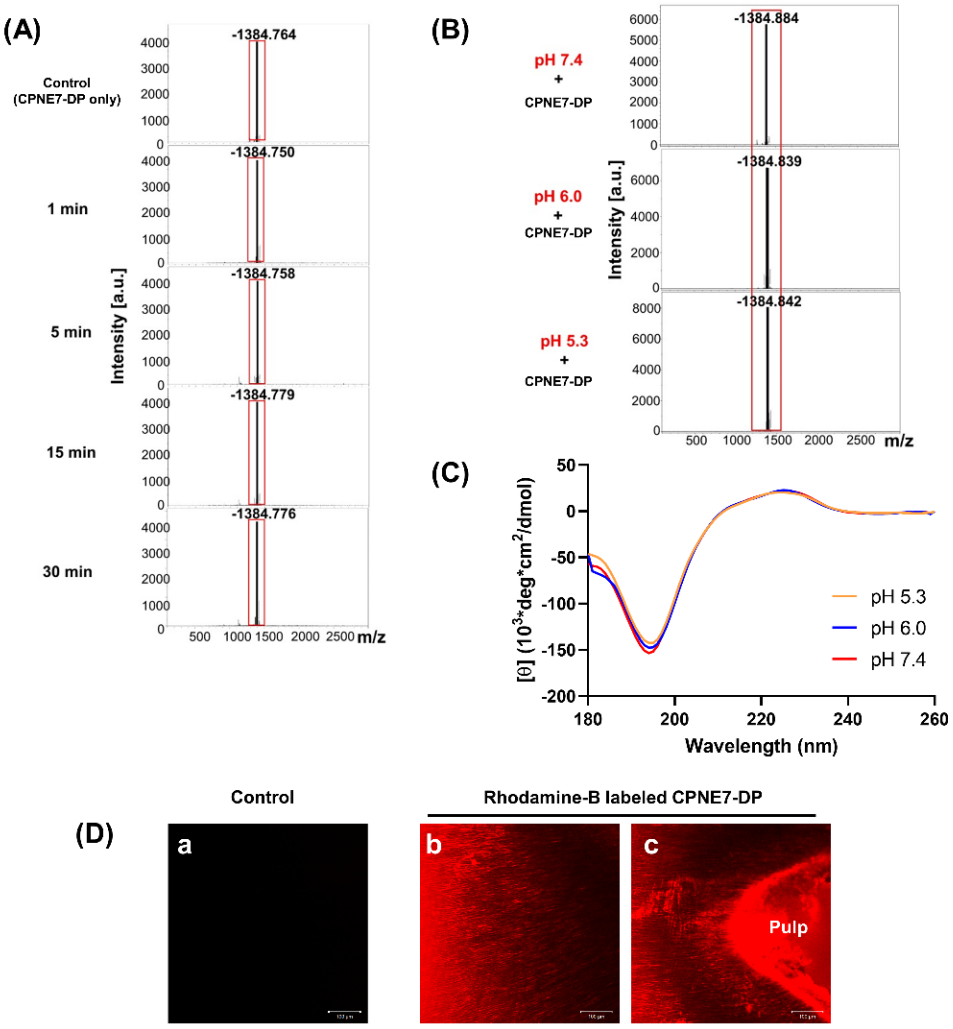

Figure 1. CPNE7-DP is stable in various stress conditions of caries lesions

(A) MALDI-TOF mass spectrometry characterization of CPNE7-DP after mixing and incubation with *S. mutans* for 1, 5, 15, or 30 min.

(B) MALDI-TOF mass spectrometry characterization of CPNE7-DP at different pH (pH 7.4, 6.0, 5.3) conditions after 30min.

(C) CD spectrum of CPNE7-DP at pH 7.4, 6.0 and 5.3 after 1 hr. The negative band of great magnitude around 200nm indicates the random coil structure. All values indicate the average of three different experiments.

(D) Evaluation of CPNE7-DP through dentinal tubules with dentin caries lesion of human extracted tooth. Confocal image of dentin and pulp after control (a) and rhodamine B-labeled CPNE7-DP (b, c) treated on dental caries. The samples were visualized using confocal laser scanning microscopy after 1 min of incubation. n=10, Scale bar = 100 μm

2. Nutrition-time/hyposalivation-based caries lesions were developed in SD rats

First, I established a nutrition-time/hyposalivation-based dental caries rat model (Figure 2A). Twenty-one rats fed with caries inducing diet showed dental caries out of twenty five. Contrary to the maxillary molars of rats fed an ordinary diet, rats fed a caries-inducing diet for 4 months exhibited dental caries. Along with the caries-inducing diet, glycopyrrolate accelerated the caries severity (Figure 2B). Additionally, the micro-CT of normal group (Figure 2C-a) showed intact molars but contrarily caries only group (Figure 2C-e) revealed cavities with destruction of the enamel and dentin.

In H&E staining, the enamel layer was removed during decalcification. The normal group (Figure 2C-b, c, d) showed undisturbed dentin with a normal odontoblast layer and pulp, while the induced-caries group (Figure 2C-f, g, h) displayed eosinophilic lesions with exposed dentinal tubules due to decalcification. Cumulatively, these results demonstrate that the nutrition-time/hyposalivation-based dentin caries lesions were successfully developed in rats.

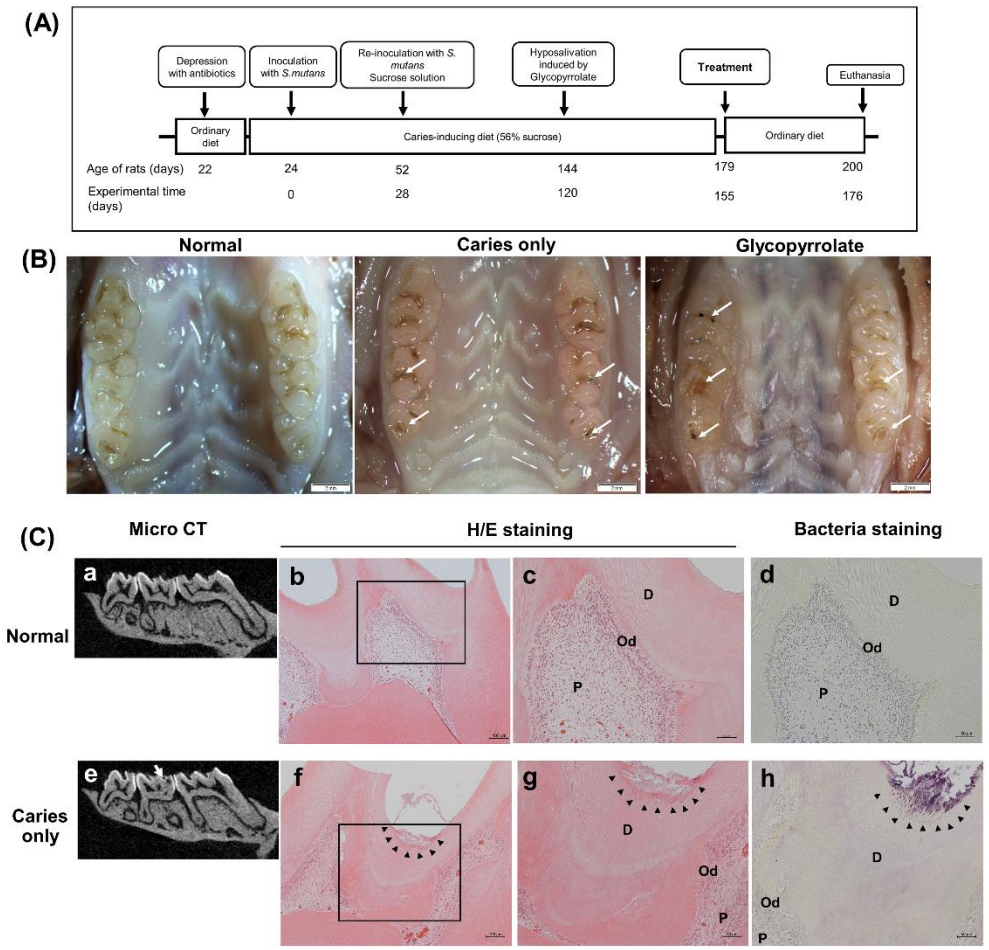

Figure 2. Nutrition-time based caries lesions were developed in rats

(A) Time schedule for nutrition-time-based caries lesions in rats.

(B) Progression of dental caries lesions in the maxilla of rats fed the caries-inducing diet. Normal, caries-inducing diet only (caries only) and after receiving glycopyrrolate for hyposalivation (glycopyrrolate) groups. White arrow indicates the caries lesion. Scale bar = 2 mm

(C) μ-CT image of the molars in the normal (a) and caries-only (e) groups. A

white arrow indicates dentin caries lesion showing low mineral density. H&E staining of normal molar (b, c) and carious lesions (f, g). Bacterial staining of normal (d) and carious lesions (h). The black arrowhead indicates the boundary of artificial dentin caries. In H&E staining, right images show the magnified region indicated by black box on the left images. Scale bar = 100μm (b, f) and 50μm (c, d, g, h).

P, pulp; Od, odontoblast; D, dentin

3. CPNE7-DP regenerated dentin and alleviated the pulp inflammation *in vivo*

To investigate the dentin-regenerating ability of CPNE7-DP, induced-caries were analyzed histologically after the topical application of CPNE7-DP on caries lesions without any surgical excavation or restoration. Based on the caries lesion depth, I classified the stage of dentin caries into two stages: early and advanced. Caries lesions up to coronal half of the total dentin thickness was classified as early caries and the deeper ones as advanced.

The untreated caries-only group exhibited infiltrating bacteria and typical aspects of pulpitis including destruction of the odontoblast layer with infiltration of inflammatory cells. Also, dentin regeneration was barely seen in this group (Figure 3A). Contrarily, the CPNE7-DP-treated groups, both early and advanced stage, showed a layer of regenerated dentin with regular dentinal tubules well laid along the coronal side of the pulp cavity (Figure 3B, C). The bacteria were found in the regenerated dentin without reaching the pulp tissue. As a result, pulp tissue was not invaded by bacteria and the inflammation was barely detectable in CPNE7-DP group. Also, the pulp of CPNE7-DP group resembled histologic features of healthy pulp with intact odontoblasts and no sign of inflammatory cells.

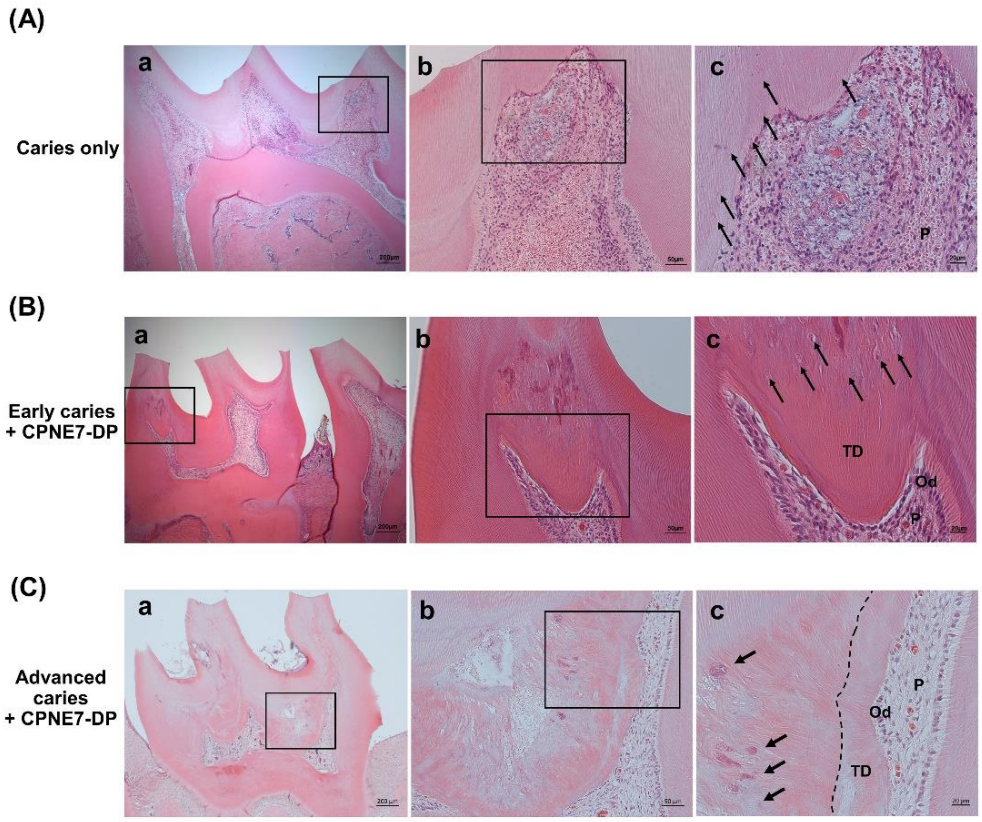

Figure 3. CPNE7-DP formed tubular dentin along the pulpal side of the dental cavities and alleviated the inflammation of the pulp in early and advanced dentin caries.

H&E staining of the rat molars in caries-only (A), CPNE7-DP treated group at early (B) and advanced (C) dentin caries. Infiltrating bacteria (black arrows) were observed in caries affected dentin. Dotted line show the boundary between caries affected and regenerative tubular dentin. Right images show the magnified region indicated by black box on the left images. Scale bar =

200μm (A-a, B-a, C-a), 50μm (A-b, B-b, C-b) and 20μm (A-c, B-c, C-c).

P, pulp; TD, tubular dentin; Od, odontoblast

4. CPNE7-DP modulated the autophagy activity in dental pulp cells

CPNE7-DP and rCPNE7 elevated CPNE7 mRNA (Figure 4A) and protein expression (Figure 4B). rCPNE7 has been reported to induce autophagy, which removes accumulated lipofuscin to reactivate odontoblasts (Y. H. Park et al., 2021). Likewise, CPNE7-DP showed autophagy modulation capacity throughout the differentiation of hDPCs. When rCPNE7 (Figure 4C-a) and CPNE7-DP (Figure 4C-b) were treated on 0 and 21 day-differentiated hDPCs, the expression of LC3, a gene marker for autophagy, was elevated. In both undifferentiated and fully differentiated hDPCs, each treatment increased LC3 expression compared to the control.

During the autophagy process, phagophore elongates to encircle cytosolic proteins and organelles to form the autophagosome. Then, the autophagosome fuse with lysosome to form the autolysosome. The number of autophagosome could be increased either by elevation of autophagosome formation or decrease of the lysosomal fusion. To clarify the autophagic stage CPNE7-DP affects, we examined the expression of markers participates in the autophagosome formation (Figure 4D). CPNE7-DP elevated the mRNA expression of Beclin-1, ATG3, ATG5 and ATG7, the representative element in the autophagosome formation.

To further clarify the effect of CPNE7-DP on autophagy level, LC3 level was tested with chloroquine which blocks the autolysosome formation (Figure 4E). LC3 level was increased by chloroquine treatment in both CPNE7-DP or LPS+CPNE7-DP group. This result further confirms that CPNE7-DP increases LC3 expression by higher level of autophagy flux, not blocking the autolysosome formation.

Finally, the LC3 induction of odontoblasts in caries lesions by CPNE7-DP was evaluated *in vivo*. LC3 positive odontoblasts were observed distinctively in the CPNE7-DP treated group (Figure 4F-d, e, f) whereas the untreated caries group did not (Figure 4F-a, b, c). These data confirms that CPNE7-DP regulates autophagy in hDPCs and pulp under caries lesion.

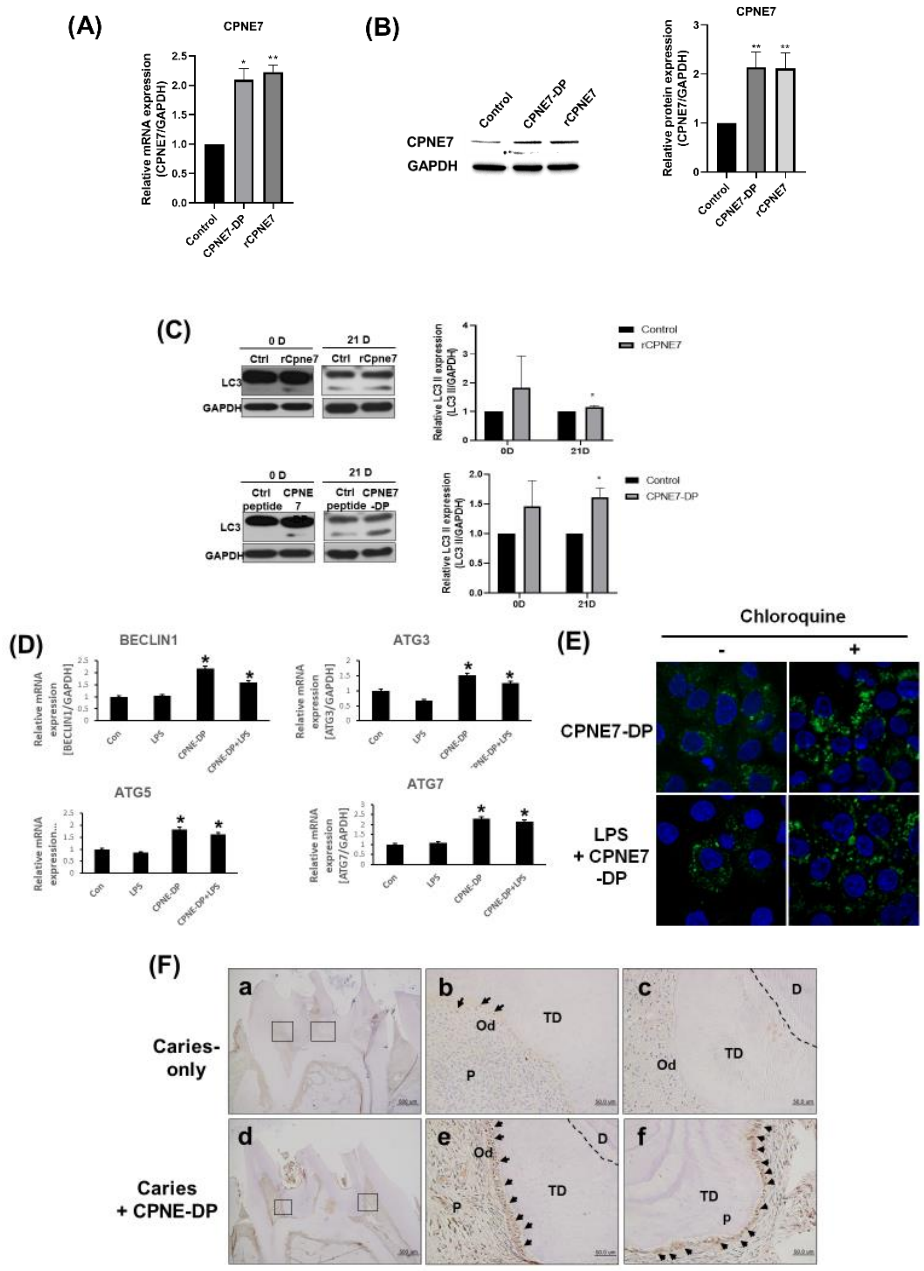

Figure 4. CPNE7-DP regenerates physiological dentin through autophagy modulation.

(A) Real-time PCR analysis of *CPNE7* mRNA in hDPCs treated with CPNE7-DP or rCPNE7. All values represent the mean ± standard deviation

of the triplicate experiments. * $p < 0.05$, ** $p < 0.01$, compared with control.

(B) Western blotting and semi-quantification analysis of CPNE7 expression in hDPCs treated with CPNE7-DP 1 μg, 10 μg, or rCPNE7. All values represent the mean ± standard deviation of the triplicate experiments. ** $p < 0.01$, compared with control.

(C) Western blot analysis of LC3 expression in rCPNE7 and CPNE7-DP treated hDPCs differentiated for 0 and 21 days. The right is the semi-quantification on relative expression of LC3 to GAPDH. All values represent the mean ± standard deviation of the triplicate experiments. * $p < 0.05$ compared to control.

(D) Real-time PCR analysis of BECLIN1, ATG3, ATG5, ATG7 mRNA in hDPCs treated with LPS, CPNE7-DP or LPS/CPNE7-DP. All values represent the mean ± standard deviation of the triplicate experiments. * $p < 0.05$, compared with control.

(E) Immunocytochemical staining for LC3 (green) and DAPI (blue) before and after chloroquine treatment in CPNE7-DP or LPS/CPNE7-DP treated hDPCs.

(F) Histological analysis of LC3 expression in the caries-only (a, b, c) and CPNE7-DP treated (d, e, f) maxillary molars. Black arrow indicates the LC3 immunoreactive cell. The dotted line indicates the boundary between dentin and regenerated tubular dentin. The right images (b, c, e, f) show the

magnified region indicated by black box on the left images (a, d). Scale bar = 500μm (a, d) and 50μm (b, c, e, f).

Od, odontoblast; TD, tertiary dentin; P, pulp; D, dentin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

5. CPNE7-DP alleviated inflammation *in vivo* and *in vitro*

Since the correlation between autophagy and pulpitis has been reported (Pei et al., 2016; Qi et al., 2019; Yang et al., 2021), I further investigated the effect of CPNE7-DP on the expression of inflammatory cytokines including TNF-α, IL-1α, IL-1β, IL-6, CXCL10, iNOS, and COX2. The expression level of inflammatory cytokines, which were elevated in the LPS-treated group, significantly decreased when CPNE7-DP and LPS were simultaneously treated. Moreover, CPNE7-DP treatment after stimulation with LPS for 24 h decreased the inflammatory cytokine levels close to that of control group (Figure 5A).

Moreover, the effect of CPNE7-DP on pulpitis was examined. The ED1 positive cells, an established indicator of inflammatory cells, were stained; the CPNE7-DP-treated group (Figure 5B-b) displayed significantly reduced ED1 positive cells in the coronal pulp compared to that in the caries-only group (Figure 5B-a). These results corroborate the anti-inflammatory effect of CPNE7-DP.

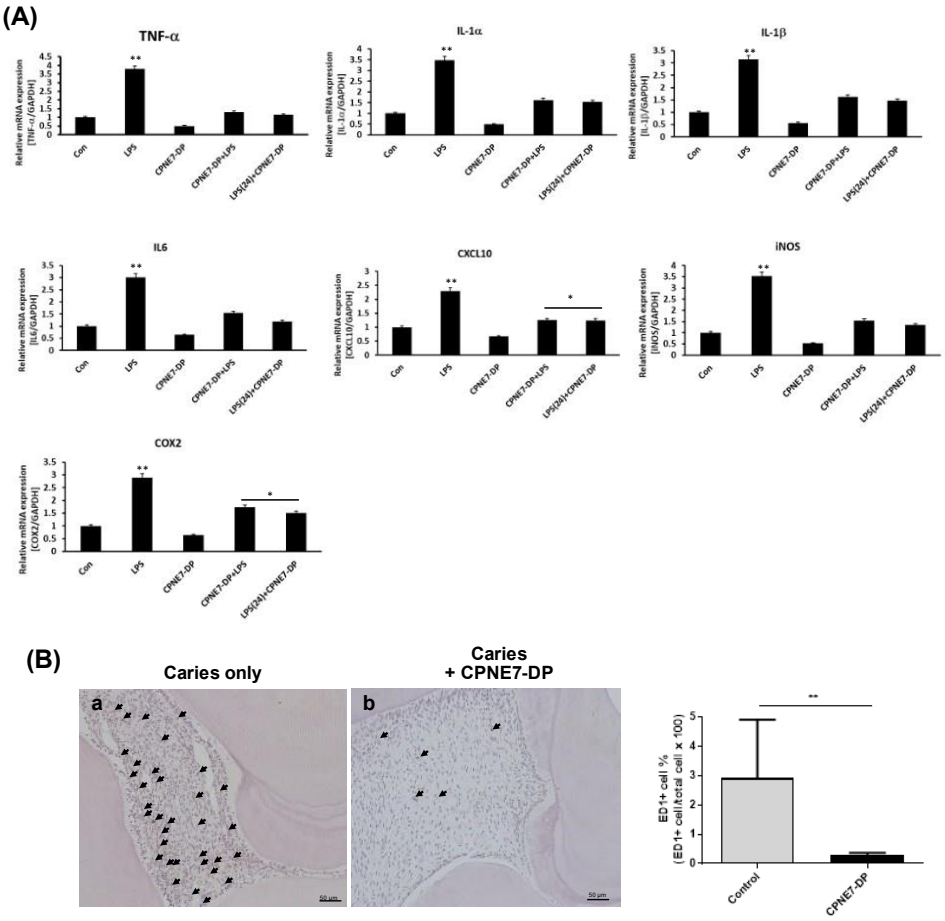

Figure 5. CPNE7-DP alleviated inflammation *in vitro* and *in vivo*.

(A) Relative expression of various inflammatory cytokines including TNF-α, IL-1α, IL-1β, IL-6, CXCL10, iNOS, and COX2 in hDPCs treated with LPS, CPNE7-DP, CPNE7-DP/LPS simultaneously and CPNE7-DP after stimulation with LPS for 24 h. All values are presented as the mean ± standard deviation. n=3. * $p < 0.05$, ** $p < 0.01$, compared to control group.

(B) Immunohistochemistry of ED1 positive with caries only (a) and caries with CPNE7-DP treatment (b). Right is the quantification of ED1 positive cell

ratio. Black arrow indicates ED1 positive cell. All values are presented as the mean ± standard deviation. 6 different teeth were measured for both group. n=6. ** $p < 0.01$, compared to control. Scale bar = 50μm

6. CPNE7-DP enhanced cell proliferation, wound healing and mineralization in inflammatory environment *in vitro*

Next, I examined the effect of CPNE7-DP on repair mechanisms. In the LPS/CPNE7-DP group, the cell survivability was significantly higher than those in the LPS-only group (Figure 6A).

Addition to the cell survivability analysis, the cell migration rate in response to the CPNE7-DP was evaluated (Figure 6B). Compared to the control group, the LPS only treated group showed more than 50% reduction of the cell migration rate; however, the treatment of CPNE7-DP rescued the cell migration rate up to the similar level as the positive control.

Moreover, the capability of CPNE7-DP to enhance mineralization under inflammatory conditions was confirmed via alizarin red staining in differentiated hDPCs (Figure 6C). CPNE7-DP increased the mineralization nodules at all differentiation timepoints, contrary to LPS; however, the number of mineralized nodules was regained by LPS/CPNE7-DP. These results suggest that CPNE7-DP might increase pulp repair capability.

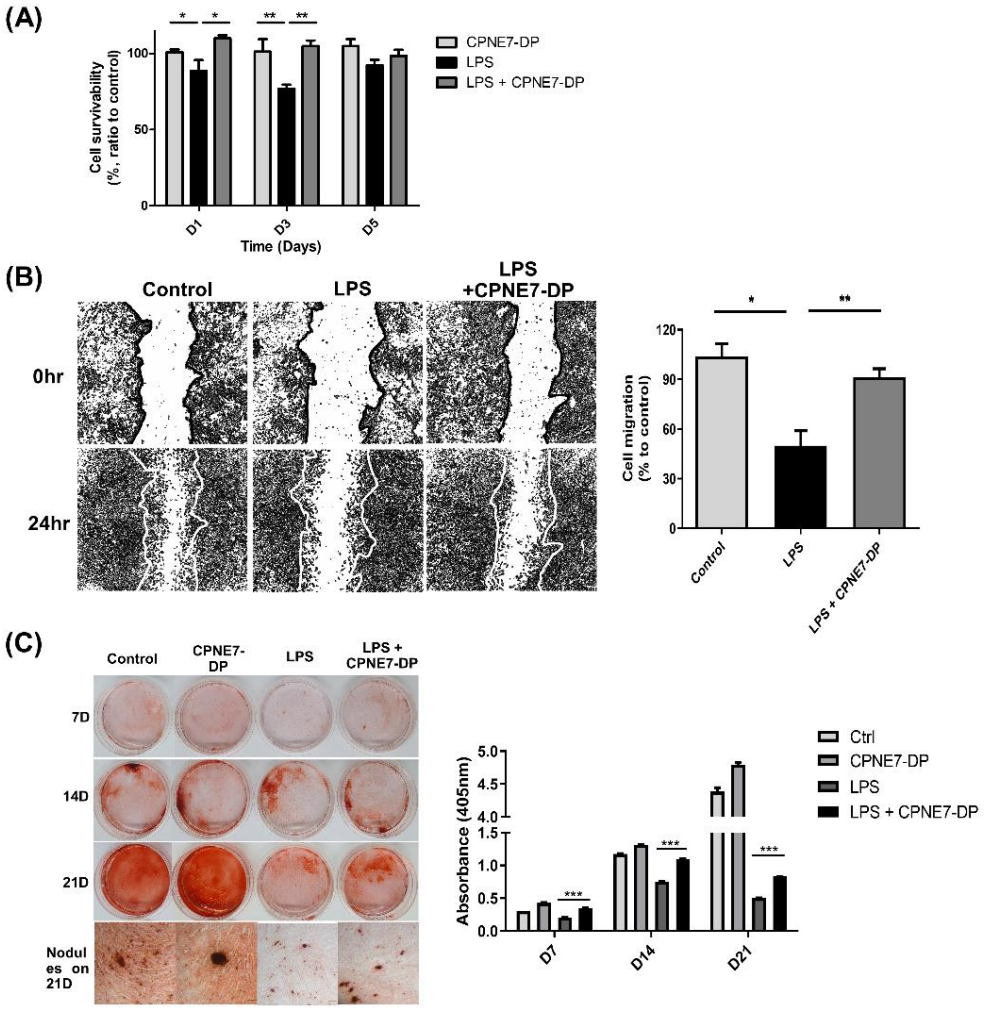

Figure 6. CPNE7-DP enhanced cell activities in an inflammatory environment *in vitro.*

(A) MTT assay for measuring the cell survivability of CPNE7-DP-, LPS-, and LPS/CPNE7-DP-treated hDPCs after indicated time. All values represent the mean ± standard deviation. (n=6) **P* < 0.05, ***P* < 0.01, compared to LPS group.

(B) Wound healing assay of Control, LPS- and LPS/CPNE7-DP-treated

hDPCs after 24 hrs. All values represent the mean ± standard deviation. (n=3) *$P < 0.05$, **$P < 0.01$, compared to LPS group.

(C) Alizarin red staining (ARS) to detect the effect of CPNE7-DP on mineralization capacity of LPS-treated hDPCs. All values represent the mean ± standard deviation. (n=3) ***$P < 0.0001$, compared to LPS group

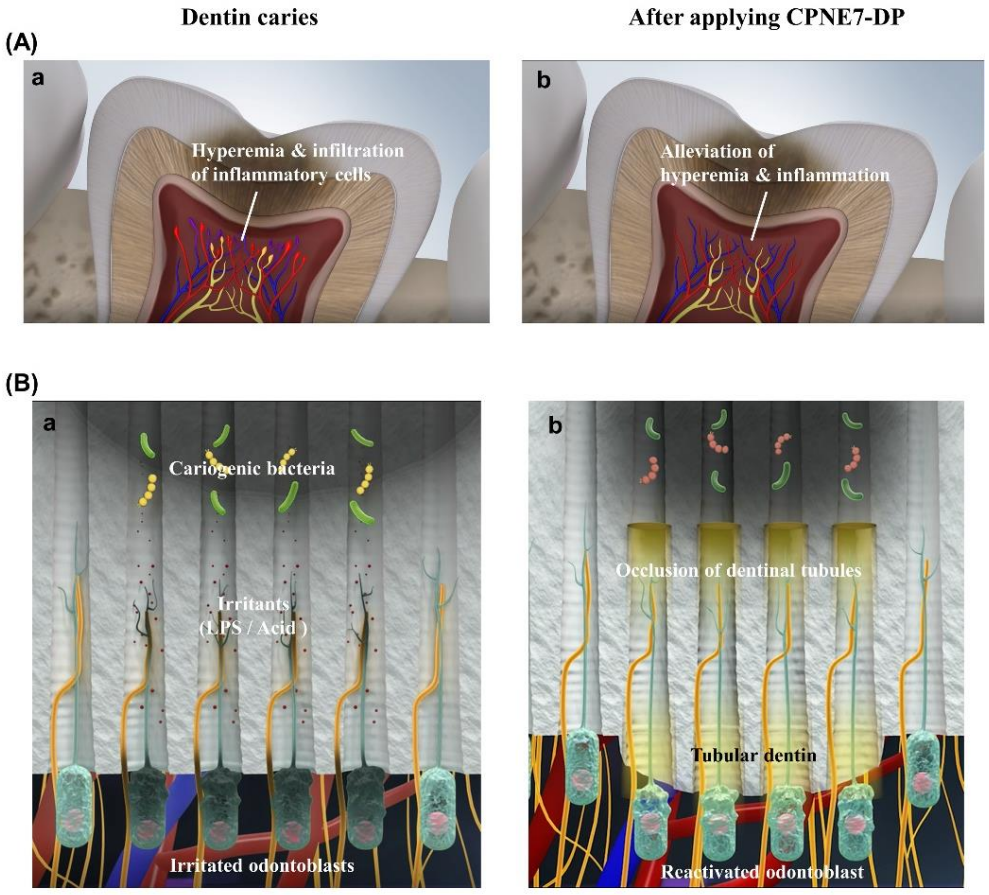

Figure 7. Schematic figure depicting dentin caries only and the CPNE7-DP treated group.

The dentin caries group (A-a) showed pulpal inflammation with hyperemia and the infiltration of inflammatory cells. After topical application of CPNE7-DP without caries removal (A-b), the pulpitis subsided with additional tubular dentin on the pulpal side. Magnified picture of the defensive front in the dentin caries group (B-a) and CPNE7-DP group (B-b). In the dentin-caries group, the bacteria irritated the odontoblast, resulting in inflammation. CPNE7-DP recovers the irritated odontoblasts to create an enclosed

environment. Then, the restored odontoblasts secrete tubular dentin to regain dentin thickness (B-b).

IV. Discussion

Dental caries is a noncommunicable disease with the highest prevalence rate, accounting for up to 5% of health expenditure regardless of a nation's industrialization (Listl et al., 2015; Petersen, 2008). Despite the prevalence of dental caries and the significance of its research, there are not many induced-caries *in vivo* models reported, especially dentin caries, due to time and labor consumption issues (Schuster et al., 1978; Yucesoy et al., 2018). Most *in vivo* models mechanically remove the enamel layer, unlike the enamel loss observed in authentic caries progression; however, I successfully developed a cost-efficient induced-caries rat model without mechanical intervention.

In an initial attempt, I explored the possibility of using beagles, which were used extensively in previous studies (H. W. Choung et al., 2016; Y. S. Lee et al., 2020) to evaluate CPNE7, as an induced-caries *in vivo* model. After 1 year of attempts, I concluded that the beagle was inappropriate for caries-related studies for the following reasons: high pH and saliva flow rate deter caries production, the coronal shape of their teeth prevent food packing, and they have different normal flora (Hale 1998; Hale 2009). However, by facilitating the development of a nutrition-time-based model, I created caries lesions in rats identical to those seen in human patients. Furthermore, considering the clinical notion that rampant caries is formed easily under

saliva-deficient conditions (Loesche et al., 1995; Samnieng, 2015), I introduced the concept of chemically reducing salivary flow rate rather than surgically excising the salivary glands. In the present study, glycopyrrolate was used in a reversible hyposalivation to promote caries progression. (Arbouw et al., 2010; Mier et al., 2000) In patients with hyposalivation, caries affect areas usually not predisposed, such as incisal edges, smooth surfaces and the lower incisor as well as other areas (Scully, 2013). Compared to previous caries *in vivo* models without the use of glycopyrrolate, my induced-caries rat model successfully and efficiently reduced the labor burden and time consumption. Through this model, which specifically allows the *in vivo* study of efficiently induced caries lesions, more extensive research on enhancing the comprehension of dental caries may be initiated in the future.

Recently, a more thorough understanding of dental caries (E. Kidd, 2000) has led to the development of novel strategies to provide more favorable options for the oral healthcare of individuals. Non-selective excavation has been considered an optimal treatment for decades. Despite many operators prioritizing traditional “restorative” dentistry, recently the idea has been challenged due to radical and extensive removal often leading to post-operative pain or inadvertent pulp exposure (Innes et al., 2016; Schwendicke et al., 2016). Underpinned by the extended comprehension of dental caries aided by improving dental adhesives, recent research supports the less invasive treatment of caries (Clarisse Abuchaim et al., 2010). Current

approaches are based on the minimally invasive dentistry (MID) concept that aims to preserve the natural tooth structure as much as possible (Peters, 2010; Rechenberg et al., 2016). However, the partial removal of the affected dentin inevitably leaves some bacterial remnants in dentinal tubule (Giacaman et al., 2018; L. Bjørndal & T. Larsen, 2000) s. After selective excavation, the pulp needs to be protected by dental adhesives for dentinal tubule sealing (Datta & BS, 2018; E. A. M. Kidd, 2004). However, as restorative materials lack long-term durability, selective excavation is merely a stopgap for the dental caries (Bergenholtz et al., 1982; Ricucci et al., 2020b). This has driven an increasing focus on biological agents to counter the limitations of anti-inflammation, restoration of tissue repair, and the disintegrated structure in caries lesions. To address these limitation, biological dentin sealing, which involves the regeneration of the tertiary dentin with an occluded external dentinal tubule surface, is gaining popularity as an optimal and practical treatment for caries (S. H. Park et al., 2019). Together with the development of dental materials, the demand for concurrent advances in bioactive materials to halt disease progression and accelerate healing is growing. From this perspective, a novel drug delivered through MID needs to restore the dentin thickness, seal the dentinal tubules, alleviate pulpitis, and reinforce the pulp's healing capacity. CPNE7-DP was stable in various acidic pH including the pH values of the active caries area, 5.01 to 5.70 (Ratanaporncharoen et al., 2018). Also, this synthetic peptide was stable with *S. Mutans* showing its potential application

for dental caries.

S. sanguinis, S. oralis, S. mitis, and *Actinomyces spp*., are known as early colonizer. These bacteria start the initial phase of biofilm formation on the acquired pellicle. With stereochemical interactions, they bind in irreversible manner to the pellicles with adhesins and fimbriae.(Marsh & Bradshaw, 1995) To facilitate the biofilm formation, these species have been inoculated with *S. mutans* in dental caries rat model.

After the construction of a caries *in vivo* model, I investigated the novel biological role of CPNE7-DP in dental caries lesions. When applied to caries lesions without any excavation or restoration, CPNE7-DP entered through the dentinal tubules and reactivated the autophagy of inflamed odontoblasts to secrete dentin matrix for physiologic dentin formation. In fact, the CPNE7-DP-treated group exhibited an alleviated extent of pulpitis compared to that in the untreated controls. Additionally, CPNE7-DP alleviated the inflammatory cytokine levels in LPS-stimulated hDPCs and promoted the repair mechanisms, such as cell viability, mineralization capacity and wound healing capacity. The anti-inflammatory effect of CPNE7-DP in simultaneously and sequentially LPS-treated hDPCs showed the possibility of both preventive and therapeutic measures. This therapeutic effect of CPNE7-DP could be a consequence of the modulation of autophagy. Consistent with a previous study, CPNE7-DP increased CPNE7 expression in the present study, which reportedly reactivates mature odontoblasts on day 21

to restore physiological functions by inducing autophagy (Y. H. Park et al., 2021). Moreover, CPNE7-DP and rCPNE7 modulated autophagy levels in hDPCs in undifferentiated hDPCs on day 0.

LPS is a bacterial toxin consisting of lipid and polysaccharide from gram-negative bacteria. Although the caries lesion includes both gram negative and positive ones, the gram-negative bacteria become more prominent as the caries progresses into dentin (Horst et al., 2009). Previous study on the bacterial population of caries showed *Veillonella* spp., which is gram-negative was the most predominant in all stages, from intact enamel to deep-dentin cavities, in both the primary and secondary dentitions (Aas et al., 2008). Also, the LPS-stimulated model is most used for pulpitis. For the above reason, I decided to use LPS-stimulated pulpitis model in this study. Considering more dominating role of *S. mutans* in dentin and deep-dentin caries of primary teeth than in those of secondary teeth, further study on LPS/*S.mutans*-stimulated pulpitis could reveal the anti-inflammatory role of CPNE7-DP in caries lesion of primary dentition.

Previous findings imply the crucial role of autophagy in pulpitis-promoting odontoblastic differentiation (Pei et al., 2016), protecting cells from pyroptosis (Kitagawa et al., 2007), promoting cell viability, and protecting cells from apoptosis pathogenesis (Wang et al., 2016). Moreover, concerning protection and adaptation, autophagy induction has advantageous effects on the differentiation and survivability of hDPCs and odontoblasts

(Yang et al., 2021). Autophagy is highly activated in irreversible pulpitis *in vivo,* especially in the odontoblast layer, and microvascular endothelial cells play a crucial role in autophagy in defense mechanisms (Qi et al., 2019). Although the therapeutic effect of CPNE7-DP treatment on caries lesions was evident, the molecular mechanism of CPNE7-DP requires further investigation.

In addition to the direct effect of CPNE7-DP on autophagy and pulpitis, an indirect effect could also be assumed. Besides the regenerated tubular dentin assisting the delivery of immune molecules to the pulp, CPNE7-DP occluded the dentinal tubule surfaces at the defect site, thereby inhibiting the flow of irritants to the pulp. To further verify the occlusion of the tubule surface by CPNE7-DP, I attempted to directly measure the nano-fluid movement of the CPNE7-DP-treated caries rat model; however, obtaining reliable results was not feasible due to the size of rats' teeth. CPNE7-DP showed no bactericidal or bacteriostatic effect in my previous experiments. (Data not shown) But still, reviewing the dentinal tubule occlusion evaluated in a previous study, enhanced mineralization in the inflammatory environment and entrapped bacteria in the induced-caries rat sufficiently mirror the biological dentin sealing capacity of CPNE7-DP (S. H. Park et al., 2019).

The rodents, including the SD rat, had a higher dentin-regenerating ability compared to humans. Even if I confirmed the reduced inflammatory

cell infiltration in the CPNE7-DP-treated rat model, the practical effectiveness of CPNE7-DP in mitigating the symptoms of caries, such as dentin sensitivity, can only be evaluated in humans. Therefore, clinical trials of CPNE7-DP in patients with dental caries remain to be conducted.

V. CONCLUDING REMARKS

In conclusion, using a newly developed cost-efficient induced-caries rat model, I evaluated the therapeutic roles of CPNE7-DP in dental caries (Figure 7). CPNE7-DP successfully produced tubular dentin and alleviated pulpitis in carious lesions. Moreover, CPNE7-DP preserved the dentin thickness with occluded dentinal tubules in the disintegrated region to serve as protection from irritants by cariogenic bacteria, thereby delaying the pulpitis progression. These results support the possibility of using CPNE7-DP as a novel bioactive molecule for MID. Furthermore, CPNE7-DP can be effectively utilized for pediatric patients in need for temporary maintenance of deciduous teeth close to exfoliation and those exhibit high anxiety over caries treatment.

REFERENCES

Aas, J. A., Griffen, A. L., Dardis, S. R., Lee, A. M., Olsen, I., Dewhirst, F. E., Leys, E. J., & Paster, B. J. (2008). Bacteria of dental caries in primary and permanent teeth in children and young adults. *Journal of Clinical Microbiology*, *46*(4), 1407–1417. https://doi.org/10.1128/JCM.01410-07

Appukuttan, D. P. M., Subramanian, S., Tadepalli, A., & Damodaran, L. K. (2015). Dental anxiety among adults: An epidemiological study in South India. *North American Journal of Medical Sciences*, *7*(1), 13–18. https://doi.org/10.4103/1947-2714.150082

Arbouw, M. E. L., Movig, P. K. L. L., Koopmann, M., Poels, P. J. E., Guchelaar, H.-J., Egberts, T. C. G., Neef, C., & van Vugt, J. P. P. (2010). Glycopyrrolate for sialorrhea in Parkinson disease A randomized, double-blind, crossover trial. *Neurology*, *74*, 1203–1207. www.controlled-trials.com.

Aubeux, D., Renard, E., Pérez, F., Tessier, S., Geoffroy, V., & Gaudin, A. (2021). Review of Animal Models to Study Pulp Inflammation. *Frontiers in Dental Medicine*, *2*. https://doi.org/10.3389/fdmed.2021.673552

Bergenholtz, G., Cox, C. F., Loesctie, W. J., & Syed, S. A. (1982). Bacterial leakage around dental restorations: its effect on the dental pulp. In *Journal of Oral Pathology* (Vol. 11).

Bortoluzzi, M., Traebert, J., Lasta, R., da Rosa, T., Capella, D., & Presta, A. (2012). Tooth loss, chewing ability and quality of life. *Contemporary Clinical Dentistry*, *3*(4), 393–397. https://doi.org/10.4103/0976-237X.107424

Caufield, P. W., Li, Y., & Dasanayake, A. (2005). Dental caries: an infectious and transmissible disease. *Compendium of Continuing Education in Dentistry (Jamesburg, N.J. : 1995)*, *26*(5 Suppl 1), 10–16.

Çehreli, Z. C., Yazici, A. R., Akca, T., & Özgünaltay, G. (2003). A morphological and micro-tensile bond strength evaluation of a single-bottle adhesive to caries-affected human dentine after four different caries removal techniques. *Journal of Dentistry*, *31*(6), 429–435. https://doi.org/10.1016/S0300-5712(03)00011-3

Choung, H. W., Lee, D. S., Lee, J. H., Shon, W. J., Lee, J. H., Ku, Y., & Park,

J. C. (2016). Tertiary Dentin Formation after Indirect Pulp Capping Using Protein CPNE7. *Journal of Dental Research*, *95*(8), 906–912. https://doi.org/10.1177/0022034516639919

Clarisse Abuchaim, Marina Rotta, Rosa Helena Miranda Grande, Alessandro Dourado Loguercio, & Alessandra Reis. (2010). Restorative Dentistry Effectiveness of sealing active proximal caries lesions with an adhesive system: 1-year clinical evaluation. *Restorative Dentistry Braz Oral Res*, *24*(3), 361–368.

Cox, C. F., White, K. C., Ramus, D. L., Farmer, J. B., & Snuggs, H. M. (1992). Reparative dentin: factors affecting its deposition. *Quintessence International (Berlin, Germany : 1985)*, *23*(4), 257–270.

Datta, S., & BS, S. (2018). SELECTIVE EXCAVATION: A PARADIGM SHIFT IN CARIOUS LESION MANAGEMENT. *International Journal of Advanced Research*, *6*(3), 1441–1444. https://doi.org/10.21474/IJAR01/6818

Dye, B. A. (2017). The Global Burden of Oral Disease: Research and Public Health Significance. In *Journal of Dental Research* (Vol. 96, Issue 4, pp. 361–363). SAGE Publications Inc. https://doi.org/10.1177/0022034517693567

Enkling, N., Marwinski, G., & Jöhren, P. (2006). Dental anxiety in a representative sample of residents of a large German city. *Clinical Oral Investigations*, *10*(1), 84–91. https://doi.org/10.1007/s00784-006-0035-6

Giacaman, R. A., Muñoz-Sandoval, C., Neuhaus, K. W., Fontana, M., & Chałas, R. (2018). Evidence-based strategies for the minimally invasive treatment of carious lesions: Review of the literature. *Advances in Clinical and Experimental Medicine*, *27*(7), 1009–1016. https://doi.org/10.17219/acem/77022

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *The Journal of Pathology*, *221*(1), 3–12. https://doi.org/10.1002/path.2697

Hale, F. (1998). Dental Caries in the Dog. *Journal of Veterinary Dentistry*, *15*(2), 79–83.

Hale, F. A. (2009). Dental caries in the dog. *Canadian Veterinary Journal*, *50*(12), 1301–1304.

Horst, O. v., Tompkins, K. A., Coats, S. R., Braham, P. H., Darveau, R. P., & Dale, B. A. (2009). TGF-β1 inhibits TLR-mediated odontoblast responses to oral bacteria. *Journal of Dental Research*, *88*(4), 333–338. https://doi.org/10.1177/0022034509334846

Innes, N. P. T., Frencken, J. E., Bjørndal, L., Maltz, M., Manton, D. J., Ricketts, D., van Landuyt, K., Banerjee, A., Campus, G., Doméjean, S., Fontana, M., Leal, S., Lo, E., Machiulskiene, V., Schulte, A., Splieth, C., Zandona, A., & Schwendicke, F. (2016). Managing Carious Lesions: Consensus Recommendations on Terminology. *Advances in Dental Research*, *28*(2), 49–57. https://doi.org/10.1177/0022034516639276

Kassebaum, N. J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C. J. L., & Marcenes, W. (2015). Global burden of untreated caries: A systematic review and metaregression. *Journal of Dental Research*, *94*(5), 650–658. https://doi.org/10.1177/0022034515573272

Kidd, E. (2000). Caries Removal and the Pulpo-dentinal Complex. *Dental Update*, *27*(10). https://doi.org/10.12968/denu.2000.27.10.476

Kidd, E. A. M. (2004). How "clean" must a cavity be before restoration? *Caries Research*, *38*(3), 305–313. https://doi.org/10.1159/000077770

Kidd, E. A., Ricketts, D. N., & Beighton, D. (1996). Criteria for caries removal at the enamel-dentine junction: a clinical and microbiological study. *British Dental Journal*, *180*(8), 287–291. https://doi.org/10.1038/sj.bdj.4809066

Kitagawa, M., Ueda, H., Iizuka, S., Sakamoto, K., Oka, H., Kudo, Y., Ogawa, I., Miyauchi, M., Tahara, H., & Takata, T. (2007). Immortalization and characterization of human dental pulp cells with odontoblastic differentiation. *Archives of Oral Biology*, *52*(8), 727–731. https://doi.org/10.1016/j.archoralbio.2007.02.006

Klinge, R. F. (2001). Further observations on tertiary dentin in human deciduous teeth. *Advances in Dental Research*, *15*, 76–79. https://doi.org/10.1177/08959374010150011901

Köhler, B., Pettersson, B. M., & Bratthall, D. (1981). Streptococcus mutans in plaque and saliva and the development of caries. *Scandinavian Journal of Dental Research*, *89*(1), 19–25. https://doi.org/10.1111/j.1600-0722.1981.tb01273.x

L. Bjørndal, & T. Larsen. (2000). Changes in the Cultivable Flora in Deep Carious Lesions following a Stepwise Excavation Procedure. *Caries*

Research, *34*, 502–508. www.karger.com/journals/cre

Lee, J. H., Lee, D. S., Choung, H. W., Shon, W. J., Seo, B. M., Lee, E. H., Cho, J. Y., & Park, J. C. (2011). Odontogenic differentiation of human dental pulp stem cells induced by preameloblast-derived factors. *Biomaterials*, *32*(36), 9696–9706. https://doi.org/10.1016/j.biomaterials.2011.09.007

Lee, Y. S., Park, Y. H., Lee, D. S., Seo, Y. M., Lee, J. H., Park, J. H., Choung, H. W., Park, S. H., Shon, W. J., & Park, J. C. (2020). Tubular dentin regeneration using a cpne7-derived functional peptide. *Materials*, *13*(20), 1–21. https://doi.org/10.3390/ma13204618

Li, F., Wang, P., Weir, M. D., Fouad, A. F., & Xu, H. H. K. (2014a). Evaluation of antibacterial and remineralizing nanocomposite and adhesive in rat tooth cavity model. *Acta Biomaterialia*, *10*(6), 2804–2813. https://doi.org/10.1016/j.actbio.2014.02.033

Li, F., Wang, P., Weir, M. D., Fouad, A. F., & Xu, H. H. K. (2014b). Evaluation of antibacterial and remineralizing nanocomposite and adhesive in rat tooth cavity model. *Acta Biomaterialia*, *10*(6), 2804–2813. https://doi.org/10.1016/j.actbio.2014.02.033

Listl, S., Galloway, J., Mossey, P. A., & Marcenes, W. (2015). Global economic impact of dental diseases. *Journal of Dental Research*, *94*(10), 1355–1361. https://doi.org/10.1177/0022034515602879

Loesche, W. J., Schork, A., Terpenning, M. S., Chen, Y.-M., & Stoll, A. J. (1995). Factors Which Influence Levels of Selected Organismsin Saliva of Older Individuals. *JOURNAL OF CLINICAL MICROBIOLOGY*, *33*(10), 4.

Marsh, P. D., & Bradshaw, D. J. (1995). Dental plaque as a biofilm. *Journal of Industrial Microbiology*, *15*(3), 169–175. https://doi.org/10.1007/BF01569822

Marshall, G. W. (1993). Dentin: microstructure and characterization. *Quintessence International (Berlin, Germany : 1985)*, *24*(9), 606–617.

Mestas, J., & Hughes, C. C. W. (2004). IMMUNOLOGY Of Mice and Not Men: Differences between Mouse and Human Immunology. In *The Journal of Immunology* (Vol. 172). http://journals.aai.org/jimmunol/article-pdf/172/5/2731/1181081/2731.pdf

Mier, R. J., Bachrach, S. J., Lakin, R. C., Barker, T., Childs, J., & Moran, M. (2000). Treatment of Sialorrhea With Glycopyrrolate A Double-blind, Dose-Ranging Study. *Arch Pediatr Adolesc Med*, *154*, 1214–1218.

Mizushima, N. (2007). Autophagy: process and function. *Genes & Development*, *21*(22), 2861–2873. https://doi.org/10.1101/gad.1599207

Nakashima, M., Iohara, K., Bottino, M. C., Fouad, A. F., Nör, J. E., & Huang, G. T. J. (2019). Animal Models for Stem Cell-Based Pulp Regeneration: Foundation for Human Clinical Applications. In *Tissue Engineering - Part B: Reviews* (Vol. 25, Issue 2, pp. 100–113). Mary Ann Liebert Inc. https://doi.org/10.1089/ten.teb.2018.0194

Park, S. H., Lee, Y. S., Lee, D. S., Park, J. C., Kim, R., & Shon, W. J. (2019). CPNE7 Induces Biological Dentin Sealing in a Dentin Hypersensitivity Model. *Journal of Dental Research*, *98*(11), 1239–1244. https://doi.org/10.1177/0022034519869577

Park, Y. H., Son, C., Seo, Y. M., Lee, Y. S., Har, A., & Park, J. C. (2021). CPNE7-Induced Autophagy Restores the Physiological Function of Mature Odontoblasts. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.655498

Pei, F., Wang, H. S., Chen, Z., & Zhang, L. (2016). Autophagy regulates odontoblast differentiation by suppressing NF-κB activation in an inflammatory environment. *Cell Death and Disease*, *7*(3). https://doi.org/10.1038/cddis.2015.397

Peters, M. C. (2010). Strategies for noninvasive demineralized tissue repair. In *Dental Clinics of North America* (Vol. 54, Issue 3). https://doi.org/10.1016/j.cden.2010.03.005

Petersen, P. E. (2008). The World Health Organization (WHO) Global Oral Health World Health Organization global policy for improvement of oral health-World Health Assembly 2007. *International Dental Journal*, *58*, 115–121. https://doi.org/10.1922/IDJ_1930Petersen07

Pohjola, V., Rekola, A., Kunttu, K., & Virtanen, J. I. (2016). Association between dental fear and oral health habits and treatment need among University students in Finland: A national study. *BMC Oral Health*, *16*(1). https://doi.org/10.1186/s12903-016-0179-y

Qi, S., Qian, J., Chen, F., Zhou, P., Yue, J., Tang, F., Zhang, Y., Gong, S., Shang, G., Cui, C., & Xu, Y. (2019). Expression of autophagy-associated proteins in rat dental irreversible pulpitis. *Molecular Medicine Reports*,

19(4), 2749–2757. https://doi.org/10.3892/mmr.2019.9944

Ratanaporncharoen, C., Tabata, M., Kitasako, Y., Ikeda, M., Goda, T., Matsumoto, A., Tagami, J., & Miyahara, Y. (2018). PH Mapping on Tooth Surfaces for Quantitative Caries Diagnosis Using Micro Ir/IrOx pH Sensor. *Analytical Chemistry*, *90*(7), 4925–4931. https://doi.org/10.1021/acs.analchem.8b00867

Rechenberg, D. K., Galicia, J. C., & Peters, O. A. (2016). Biological markers for pulpal inflammation: A systematic review. In *PLoS ONE* (Vol. 11, Issue 11). Public Library of Science. https://doi.org/10.1371/journal.pone.0167289

Ricucci, D., Siqueira, J. F., Rôças, I. N., Lipski, M., Shiban, A., & Tay, F. R. (2020a). Pulp and dentine responses to selective caries excavation: A histological and histobacteriological human study. *Journal of Dentistry*, *100*, 103430. https://doi.org/10.1016/j.jdent.2020.103430

Ricucci, D., Siqueira, J. F., Rôças, I. N., Lipski, M., Shiban, A., & Tay, F. R. (2020b). Pulp and dentine responses to selective caries excavation: A histological and histobacteriological human study. *Journal of Dentistry*, *100*. https://doi.org/10.1016/j.jdent.2020.103430

Samnieng, P. (2015). Association of Hyposalivation with Oral Function, Nutrition, and Oral Health in Visual Impaired Patient. *International Journal of Clinical Preventive Dentistry*, *11*(1), 15–20. https://doi.org/10.15236/ijcpd.2015.11.1.15

Schuster, G. S., Navia, J. M., Amsbaugh, S., & Larson, R. H. (1978). Sources of Variability in Rat Caries Studies: Microbial Infection and Caging Procedure. *Journal of Dental Research*, *57*(2), 355–360.

Schwendicke, F., Frencken, J. E., Bjørndal, L., Maltz, M., Manton, D. J., Ricketts, D., van Landuyt, K., Banerjee, A., Campus, G., Doméjean, S., Fontana, M., Leal, S., Lo, E., Machiulskiene, V., Schulte, A., Splieth, C., Zandona, A. F., & Innes, N. P. T. (2016). Managing Carious Lesions: Consensus Recommendations on Carious Tissue Removal. *Advances in Dental Research*, *28*(2), 58–67. https://doi.org/10.1177/0022034516639271

Scully, C. (2013). *Oral and Maxillofacial Medicine* (3rd ed.).

Seo, Y. M., Park, S. J., Lee, H. K., & Park, J. C. (2017). Copine-7 binds to the cell surface receptor, nucleolin, and regulates ciliogenesis and Dspp expression during odontoblast differentiation. *Scientific Reports*, *7*(1).

https://doi.org/10.1038/s41598-017-11641-y

Smith, A. J., Cassidy, N., Perry, H., Bègue-Kirn, C., Ruch, J. v, & Lesot, H. (1995). Reactionary dentinogenesis. *The International Journal of Developmental Biology*, *39*(1), 273–280.

Tomsig, J. L., Snyder, S. L., & Creutz, C. E. (2003). Identification of targets for calcium signaling through the copine family of proteins. Characterization of a coiled-coil copine-binding motif. *The Journal of Biological Chemistry*, *278*(12), 10048–10054. https://doi.org/10.1074/jbc.M212632200

Vujicic, M., Buchmueller, T., & Klein, R. (2016). Dental care presents the highest level of financial barriers, compared to other types of health care services. *Health Affairs*, *35*(12), 2176–2182. https://doi.org/10.1377/hlthaff.2016.0800

Wang, H. S., Pei, F., Chen, Z., & Zhang, L. (2016). Increased apoptosis of inflamed odontoblasts is associated with CD47 loss. *Journal of Dental Research*, *95*(6), 697–703. https://doi.org/10.1177/0022034516633639

Williams, S., Wei, L., Griffin, S. O., & Thornton-Evans, G. (2021). Untreated caries among US working-aged adults and association with reporting need for oral health care. *Journal of the American Dental Association*, *152*(1), 55–64. https://doi.org/10.1016/j.adaj.2020.09.019

World Health Oraganization. (2017). World Health Statistics. *World Health Statistics*. https://apps.who.int/iris/handle/10665/255336

Yang, S., Fan, W., Li, Y., Liu, Q., He, H., & Huang, F. (2021). Autophagy in tooth: Physiology, disease and therapeutic implication. In *Cell Biochemistry and Function* (Vol. 39, Issue 6, pp. 702–712). John Wiley and Sons Ltd. https://doi.org/10.1002/cbf.3636

Yucesoy, D. T., Fong, H., Gresswell, C., Saadat, S., Chung, W. O., Dogan, S., & Sarikaya, M. (2018). Early Caries in an In Vivo Model: Structural and Nanomechanical Characterization. *Journal of Dental Research*, *97*(13), 1452–1459. https://doi.org/10.1177/0022034518789898

Zero, D. T., Zandona, A. F., Vail, M. M., & Spolnik, K. J. (2011). Dental caries and pulpal disease. *Dental Clinics of North America*, *55*(1), 29–46. https://doi.org/10.1016/j.cden.2010.08.010

VI. ABSTRACT IN KOREAN

CPNE7 펩타이드의 생리적 상아질 형성을 통한 치아 우식증의 새로운 치료 방법

CPNE7 단백질에서 유래한 기능성 펩타이드인 CPNE7 펩타이드는 상아질 관련 질환을 위한 생리활성 물질로 연구되어 왔다. CPNE7 펩타이드를 상아질 손상부위에 도포하면 치수 쪽에는 세관 상아질을 형성할 뿐만 아니라 상아세관 또한 막는 효과를 보인다. CPNE7 펩타이드는 보통 치경부 마모증을 동반하는 치아지각과민증에 대해 효과를 보인 바 있다. 그러나 또 다른 다빈도 상아질 질환인 상아질 치아우식증에 대한 CPNE7 펩타이드의 효과는 연구된 바가 없다. 이에 이번 연구에서는 랫드에서 치아우식증을 유도한 후 CPNE7 펩타이드의 치아우식증 진행정지 효과를 확인하고자 한다.

먼저 치아우식증의 다양한 조건들에서 CPNE7 펩타이드의 안정성을 확인하였다. 이러한 조건들에는 치아우식증의 원인균과 낮은 산도가 있다. 또한 생체 내에서의 효과를 정확히 알아보기 위해 장기 우식증 유발 섭식/타액분비저하 치아우식증 랫드 모델을 개발하였다. 이 모델은 사람의 치아우식증을 최대한 재현하기

위해 치아우식증 원인균을 랫드의 구강에 접종하고 치아우식 유발 사료를 충분한 시간 동안 급여하였다. 글리코피롤레이트를 적용하여 일시적인 타액분비저하를 일으켜 치아우식증을 촉진하였다. 유발된 치아우식증에CPNE7 펩타이드를 적용한 후 조직학적 평가를 통해 세관 상아질의 형성을 확인하였다. 또한CPNE7 펩타이드 항염증 효과를 살펴보기 위해 사람치수세포 및 동물 모델 상에서 염증 마커 유전자의 발현의 변화를 조사하였다.

CPNE7 펩타이드는 치아우식증 원인균 및 치아우식증의 낮은 pH에서 안정하였다. 앞서 언급한 랫드 모델에서 치아우식증의 발생은 방사선학적 방법 및 조직학적 방법으로 확인되었다. 치아우식증 동물 모델에서 CPNE7 펩타이드는 상당량의 세관 상아질을 재생시켰고 치수염을 완화시켰다. 사람치수세포를 사용한 치수염 세포 실험에서 CPNE7 펩타이드는 염증 인자의 발현량을 줄이는 효과도 보였다. 이러한 현상들은 CPNE7 펩타이드의 자가포식작용의 조절을 통한 염증상태의 상아모세포 재활성화의 결과일 것으로 추정된다.

종합해 볼 때, CPNE7 펩타이드는 생리적 상아질 형성을 통해 치아우식증의 진행을 정지하며, 이러한 효과는 현재 시행되는 치아 우식의 수복 방법들의 한계점을 보완할 수 있을 것으로 예상된다.

주 요 어: 재생의학, 최소침습치의학, 치아우식증 치료법, 상아모세포, 생리활성물질, 치수염

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