Comparison of Biocompatibility of Newly Developed Calcium Silicate-Based Sealers and Epoxy Resin-Based Sealer on Human Periodontal Ligament Stem Cells

Hanseul Oh

The Graduate School

Yonsei University

Department of Dentistry

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This certifies that the Master's Thesis of

Hanseul Oh is approved.

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Euiseong Kim

_____

Su-Jung Shin

_____

Sunil Kim

The Graduate School

Yonsei University

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Abstract

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Hanseul Oh, D.D.S.

Department of Dentistry

The Graduate School, Yonsei University

(Directed by Professor Euiseong Kim, D.D.S., M.S.D., Ph.D.)

The aim of this study was to evaluate various biocompatibility of newly introduced calcium silicate-based sealers (CeraSeal and EndoSeal TCS) and epoxy resin-based sealer (AH-Plus) on various aspect on human periodontal ligament stem cells (hPDLSCs).

hPDLSCs were acquired from premolars (n = 4) of four subjects, whose ages extended from 16 to 24 years of age. To make media extracted from freshly mixed sealers (fresh

media), all unset experimental sealers were mixed with culture medium (DMEM) and incubated for 24 h. To make media extracted from set sealers (setting media), materials were set in disc form for 48 h, and then extracted in DMEM for 24 h. hPDLSCs were cultured in fresh media or setting media according to each experimental condition. The expression of mesenchymal stem cell surface molecules (CD11b, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR) was analyzed with flow cytometry (FACS) after culture in setting media. Cell viability was assessed using cell viability assay (Cell Counting Kit-8; CCK-8) after culture in fresh and setting media. To evaluate inflammatory response to the materials, concentrations of IL-6, IL-8, TGF-β in fresh and setting media were analyzed using ELISA kit. The osteogenic potential of hPDLSCs in setting media was quantified with RT-qPCR (Real Time-quantitative Polymerase Chain Reaction) for ALP, OCN, and RUNX2, and visually qualified with ALP (Alkaline Phosphatase) staining and ARS (Alizarin Red S) staining. Cell attachment on material and material surface morphology was evaluated with Scanning Electronic Microscopy. Statistical differences were assessed by analysis of variance followed by the Tukey's test ($p < 0.05$).

In FACS analysis, mesenchymal stem cell markers showed high level (> 99%) and the hematopoietic markers showed low expression level (<1%) in both Calcium silicate-based sealers and AH-Plus, therefore stemness of hPDLSCs was maintained in all materials. In cell viability test, AH-Plus showed the lowest cell viablity in all experimental periods, and CeraSeal showed significantly higher cell viability than others in fresh media ($p < 0.05$). In setting media, cell viablity was not significantly different between materials over all time

periods. In ELISA test, AH-Plus showed higher expression of pro-inflammatory cytokines (IL-6 and IL-8) than other sealers. In the anti-inflammatory cytokine TGF-β, only CeraSeal maintained a similar level to control in setting media. In RT-qPCR test, AH-Plus showed lower expression level than other material, however EndoSeal TCS showed better expression level than others. As a result of ALP and ARS staining tests, calcium silicate-based sealers were stained similarly to positive control, and AH-Plus was less stained. Finally, scanning electron microscopy studies showed low degree of cell proliferation and differentiation on AH-Plus. However, CeraSeal and EndoSeal TCS showed high degree of cell proliferation and differentiation.

Calcium silicate-based sealers are more biocompatible and less cytotoxic than epoxy-resin based sealer. In particular, CeraSeal showed less cytotoxicity than other materials before setting, and EndoSeal TCS showed better osteogenic potential than other materials.

Keywords : biocompatibility; root canal sealer; calcium silicate; epoxy resin; cell viability; osteogenic differentiation; SEM

Comparison of Biocompatibility of Newly Developed Calcium Silicate-Based Sealers and Epoxy Resin-Based Sealer on Human Periodontal Ligament Stem Cells

Hanseul Oh

Department of Dentistry
The Graduate School, Yonsei University
(Directed by Professor Euiseong Kim)

I. Introduction

The purpose of the root canal obturation is to form a tight barrier to protect the apical tissue from various microorganisms in the oral cavity (Tomson et al., 2014). The previous

effort to form a tight barrier was to fill the root canal with gutta percha as much as possible, provided that the gutta percha has better properties and biocompatibility than the sealer (Cohen and Hargraves, 2010; Pascon and Spngberg, 1990; Tomson et al., 2014). To achieve that, various filling methods developed from the lateral condensation to the continuous wave compaction technique (Cohen and Hargraves, 2010). However, with the advent of bioceramic sealers such as calcium silicate-based sealers, the single cone technique was recommended, and accordingly the amount of sealer entering the root canal increased (Donnermeyer et al., 2018). In particular, since most calcium silicate-based sealer products are provided in the form of prefilled syringes, clinicians inject sealers directly using a syringe needle in the root canal (Tomson et al., 2014). In this case, there is a higher probability that the sealer is extruded beyond the root canal space or the sealer puff occurs compared to the conventional techniques (Lee et al., 2019).

Preferably, root canal filling should be terminated at the physiological apical foramen (Cohen and Hargraves, 2010). However, if the sealers are extruded beyond the physiological apical foramen, the unset sealers can irritate the periapical tissues (Scarparo et al., 2009). This irritation can also occur through the accessory canals or dentinal tubules, and even in the absence of extrusion, the sealers can release soluble toxic substances in the periapical tissues (Cohen and Hargraves, 2010; Victoria-Escandell et al., 2017). This may affect bone metabolism and healing in the periapical tissues (Victoria-Escandell et al., 2017). In addition, if the sealers have osteogenic potential, apical healing can be promoted after canal obturation. Therefore, it is necessary to evaluate the biocompatiblity and

osteogenic potential of sealers for hPDLSCs.

In the previous study (Lee et al., 2019), some calcium silicate-based sealers (EndoSeal MTA, Nano-ceramic Sealer and Wellroot ST) were compared with the epoxy resin-based sealers (AH-Plus and AD Seal), and the results confirmed that the calcium silicate-based sealers had better biocompatibility than epoxy resin-based sealers. As the development of new calcium silicate-based sealers, compositional change of sealers could alter their biological characteristics and bioactivity (Collado-González et al., 2017). Therefore, the study to evaluate their biocompatibility is needed if a new calcium silicate based sealer is introduced. In this study, experiments were conducted on newly introduced calcium silicate-based sealers (CeraSeal; Meta-Biomed, Cheongju, Korea; EndoSeal TCS; Maruchi, Wonju, Korea). CeraSeal is composed of tricalcium silicates, dicalcium silicates, calcium aluminates, zirconium oxides, and thickening agents. It is said that synthesized pure calcium silicate compound was used. EndoSeal TCS is a developed product from EndoSeal MTA (Maruchi, Wonju, Korea), consisting of tricalcium silicates, phyllosilicate minerals, zirconium oxides, and dimethyl sulfoxides. It is said that pure tricalcium silicate was synthesized and used without using the portland cement, which is the main component of EndoSeal MTA. However, there are rare studies regarding the biocompatibility of these newly developed calcium silicate-based sealers. The study to evaluate their biocompatibility was needed. In this study, I tested various biocompatibility of newly introduced calcium silicate-based sealers in terms of cell viability, inflammatory response, expression of mesenchymal phenotype, osteogenic potential, cell attachment and

morphology.

II. Materials and methods

1. Isolation and culture of hPDLSCs

hPDLSCs were acquired from premolars (n = 4) of four subjects, whose ages extended from 16 to 24 years of age. Teeth were extracted for orthodontic treatment plan, and the consent forms according to the guidelines of the Ethics Committee of the research institution (Yonsei University Dental Hospital Institutional Review Board number: 2-2017-0002) were acquired from the donors. hPDL tissue was obtained from the mid-third of the root and minced into small pieces with a surgical blade no.10. After that, the pieces of hPDL tissue were washed multiple times with Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) including 3% penicillin-streptomycin (Gibco) and put on a 6-well culture plate (SPL, Pocheon, Korea). The tissues were cultured in DMEM with 15% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin. Migrated hPDLSCs from the explants were passed on to passage 2 by cell separation with trypsin (Gibco). The separated hPDLSCs were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (normal culture media) in 5% CO2 at 37 °C. hPDLSCs at passages 4 to 6 were used for all experiments.

2. Preparation of Fresh/Setting Material Extraction Medium

Two calcium silicate-based sealers (CeraSeal; Meta-Biomed, Cheongju, Korea; EndoSeal

TCS; Maruchi, Wonju, Korea) and an epoxy resin-based sealer (AH-Plus; DentsplySirona, Tulsa, OK, USA) were used for the experimental materials (Figure 1, Table 1).

Figure 1. Tested sealers in this study. (A) CeraSeal, (B) EndoSeal TCS, (C) AH-Plus

Table 1. The information of the tested sealers in this study

Sealer	Manufacturer	Composition	
AH-Plus	DentsplySirona, Tulsa, OK, USA	***Paste A*** 25-50% bisphenol A 10-25% zirconium dioxide NS calcium tungstate NS iron oxide	***Paste B*** 2.5-10% N,n-dibenzyl-5-oxanonandiamin-1,9 2.5-10% amantadine
CeraSeal	MetaBiomed, Cheongju, Korea	Tricalcium silicate, Dicalcium silicate, Calcium aluminate, Zirconium oxide, Thickening agent	
EndoSeal TCS	MARUCHI, Wonju, Korea	Tricalcium silicate, Phyllosilicate mineral, Zirconium oxide, Dimethyl sulfoxide	

To make fresh material extraction medium (fresh medium), all unset experimental sealers were prepared and mixed with culture medium (DMEM) at a concentration of 20 mg/mL, incubated at 37 °C in 5% CO_2 for 24 h. The supernatant of fresh material extraction medium

was filtered with a 0.2 μm pore-size syringe filter (Corning; Merck, Darmstadt, Germany). To make setting material extraction medium (setting medium), all sealers were mixed and prepared using instructions of the manufacturers and incubated at 37 °C in 100% relative humidity for 48 h to permit them to be set completely. Set materials were made in the form of discs (5-mm diameter and 2-mm thick, 200 mg weight) created from tube shaped Teflon molds in sterile conditions. Each set sample disc was stored in 10ml DMEM solution with 1% penicillin-streptomycin and 10% FBS (normal culture media) at 37 °C in 5% CO_2 for 24h and filtered through a 0.2 μm filter (Minisart, Goettingen, Germany) (Figure 2).

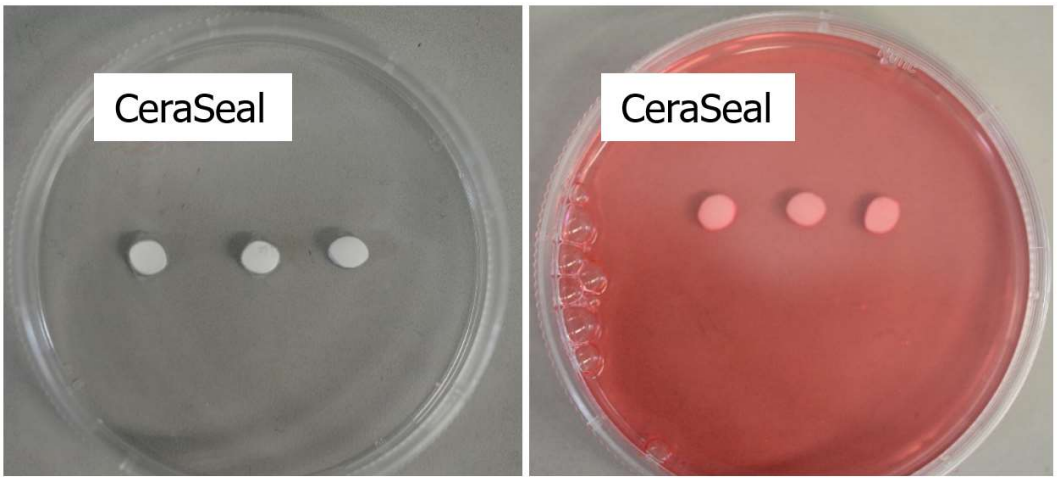

Figure 2. Example of set material discs (Left) and extracted medium (Right). Each set material disc was stored in normal culture media for 24 hours.

3. Measurement of Mesenchymal Phenotype by Flowcytometry Analysis (FACS)

The expression of mesenchymal stem cell surface molecules was analyzed with flow cytometry (FACS verse; BD bioscience) assay. hPDLSCs (1×10^5 cells) were seeded in a 6-well plate, and then after 24 h the medium was changed to setting extraction medium and incubated for 3 days and 7 days. The cells were treated with 0.05% Trypsin-EDTA, PBS washing, finally made a single-cell suspension for FACS analysis. The CD markers antigen-antibody reaction in hPDLSCs was examined with BD Stemflow human MSC analysis kit (BD Biosciences, Piscataway, NJ, USA). Target surface markers include the typical mesenchymal stem cell markers (CD90, CD105, and CD73) and the hematopoietic markers (CD11b, CD19, CD34, CD45, and HLA-DR). The data were analyzed with FlowJo (BD Biosciences).

4. Cell Viability Assay (CCK-8)

To evaluate the cytotoxicity of the unset sealer and the fully set sealer for hPDLSC, cell viability test was performed with fresh media and setting media respectively using Cell Counting Kit-8 assay. The hPDLSCs (1×10^4 cells per well) were seeded in a 96-well plate and exposed to the fresh and setting material extraction media. After 1 day, 3 days and 7 days, cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's guidelines. The absorbance at 450nm was measured using the spectrophotometer (VersaMaxMultiplate

Reader, Thermo Fisher Scientific, Waltham, MA, USA). Control group (normal hPDLSCs) was cultured in normal culture media with the same time as the experimental groups without media change to experimental media and evaluated.

5. Evaluation of Inflammatory Response by Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was done to examine the inflammation response of materials. hPDLSCs (1×10^5 cells) were seeded in a 6-well plate, and then after 24 h the medium was changed to fresh or setting extraction medium and incubated for 24h. I examined IL-6 and IL-8 as pro-inflammatory cytokines, and examined TGF-β as anti-inflammatory cytokine. The concentrations of IL-6, IL-8, TGF-β in the fresh extraction medium were analyzed using ELISA kit according to the manufacturer's guidline (R&D systems, Minneapolis, MN, USA) and measured in a microplate reader (BioTek, Winooski, VT, USA) at 450 nm. Control group (normal hPDLSCs) was cultured in normal culture media with the same time as the experimental groups without media change to experimental media and evaluated.

6. Quantification of Osteogenic potentials by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

hPDLSCs were cultured for 3 and 7 days in setting media including osteogenic-inducing reagents (10 mM b-glycerol phosphate, 100 μM l-ascorbic acid 2-phosphate, and 10 nM dexamethasone) in order to examine the pattern of osteogenic markers for all sealers using real time-quantitative polymerase chain reaction (RT-qPCR). To examine the effects of sealers on osteoblastic differentiation, the mRNA expression levels of osteocalcin (OCN), alkaline phosphatase (ALP), and runt-related transcription factor 2 (RUNX2) was examined using β-actin gene as endogenous control. Isolation of mRNA and transcription into cDNA was done with the RNeasy mini kit (Qiagen) and RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific). qPCR was done using the Taqman gene expression assays(RUNX2, Hs01047943_m1; ALP, Hs00758162_m1; OCN; Hs01587814_g1, and β-actin, Hs01060665_g1) with the QuantStudio 3 RT-qPCR systems (Applied Biosystems, Foster City, CA, USA). The gene expression level of OCN, ALP, and RUNX2 was analyzed using this method. Control group (osteoinducted hPDLSCs) was cultured in osteoinduction media (MEM + osteogenic-inducing reagents) with the same time as the experimental groups and evaluated.

7. Alkaline Phosphatase (ALP) and Alizarin Red S (ARS) Staining

Alkaline phosphatase (ALP) staining and Alizarin Red S (ARS) staining was conducted to examine the osteogenic activity. For ALP staining, hPDLSCs (2.5×10^4 cells) were placed in a 24-well plate and incubated for 3 and 7 days in the setting media with osteogenic-inducing reagents (10 mM b-glycerol phosphate, 100 μM l-ascorbic acid 2-

phosphate, and 10 nM dexamethasone). After each time period, cells were fixed with 4% PFA, washed in PBS, and stained using the ALP Staining Kit (Merck). For ARS staining, hPDLSCs (2.5×10^4 cells) were incubated for 14 days in the setting media with osteogenic-inducing reagents (10 mM b-glycerol phosphate, 100 μM l-ascorbic acid 2-phosphate, and 10 nM dexamethasone). After 14days, cells were fixed with 4% PFA, washed in PBS, and stained with the 2% ARS solution (Acros, Gyeonggi-do, Korea). The ALP and ARS stained specimen photographed using optical microscope (H.K 3.1, Koptic, Gyeonggi-do, Korea) and digital camera (Nikon, Tokyo, Japan). Negative control groups (normal hPDLSCs) was cultured in normal culture media and positive control groups (osteoinducted hPDLSCs) was cultured in osteoinduction media with the same time as the experimental groups, stained and evaluated with the same protocol as above.

8. Evalulation of cell attachment and material surface morphology by Scanning Electronic Microscopy

All set materials were made in the form of discs (5-mm diameter and 2-mm thick, 200 mg weight) and incubated for 48 h to permit them to be set completely in an incubator (37 °C, 100% relative humidity). After setting for 48 h, hPDLSCs (5×10^4 cells/mL) were seeded onto each set disc directly and incubated at 37 °C in 5% CO_2 for 72 h. The cultured discs were washed with PBS and fixed with 2% glutaraldehyde. All discs were dehydrated, air-dried, and 100-nm-thick Au/Pd coated to examine the cell attachment and morphology using field emission scanning electronic microscopy (FE-SEM; Merlin, Carl ZEISS,

Germany). Material surface morphology was examined after 48 h material setting without hPDLSCs seeding in the same protocol as above.

9. Statistical Analysis

All experiments were repeated at least three times per cell line (n = 4). Differences among material groups were evaluated using the IBM SPSS statistics 25.0 software (IBM, Armonk, NY, USA) with one-way analysis of variance followed by the Tukey's test. To indicate statistical significance, $p < 0.05$ was considered.

III. Results

1. Measurement of Mesenchymal Phenotype by Flowcytometry Analysis (FACS)

Throughout all experimental groups including AH-Plus, their expression of the typical mesenchymal stem cell markers (CD90, CD105, and CD73) showed high level (> 99%) (Figure 3). However the hematopoietic markers (CD11b, CD19, CD34, CD45, and HLA-DR) showed low expression level (< 1%).

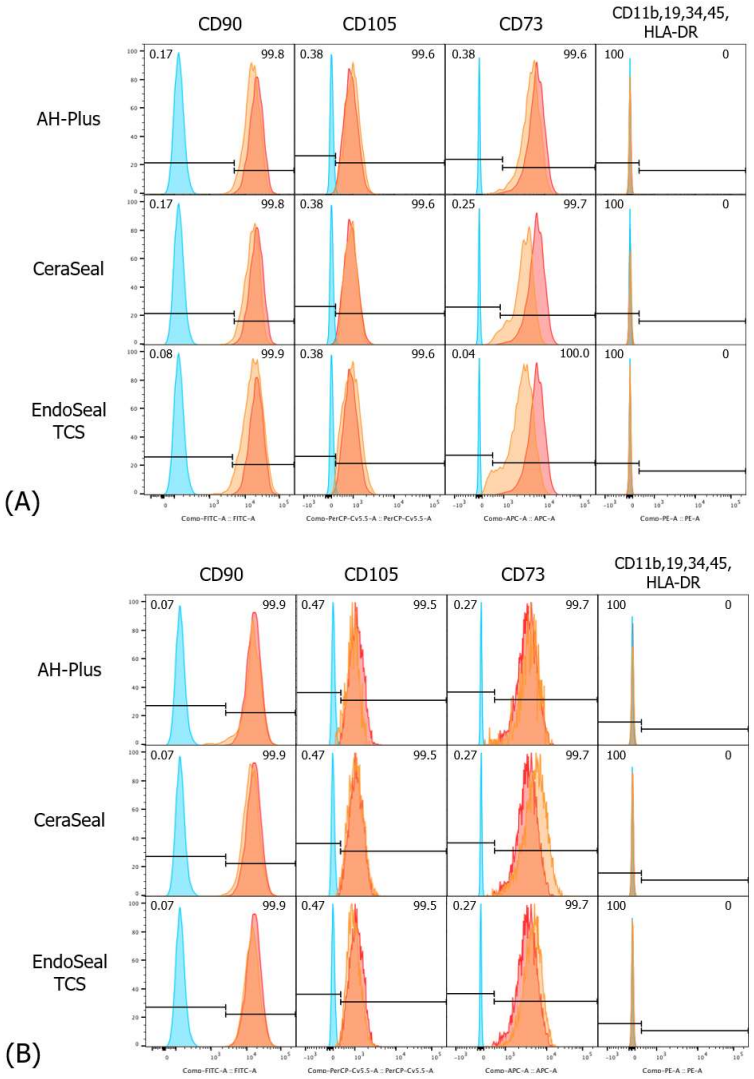

Fig 3. Mesenchymal phenotype expression of hPDLSCs analyzing with flow cytometry(FACS) after culture in setting media for (A) 3days, (B) 7days. Cell surface markers of hPDLSCs, including CD90, CD105, CD73, CD11b, CD19, CD34, CD45, and HLA-DR were analyzed with flow cytometry. Blue tracing, Isotype; Orange tracing, experimental group; Red tracing, normal group cultured in normal culture media.

2. Cell Viability Assay (CCK-8)

In fresh media, AH-Plus showed the lowest cell viablity in all experimental periods (Figure 4). Calcium silicate-based sealers showed similar viability to control (cultured in normal culture media) at Days 1 and 3, whereas at Day 7, cell viabiliy of CeraSeal significantly increased compared to control and EndoSeal TCS (Figure 4A). In setting media, on the other hand, cell viablity of hPDLSCs was not significantly different between materials over all time periods.

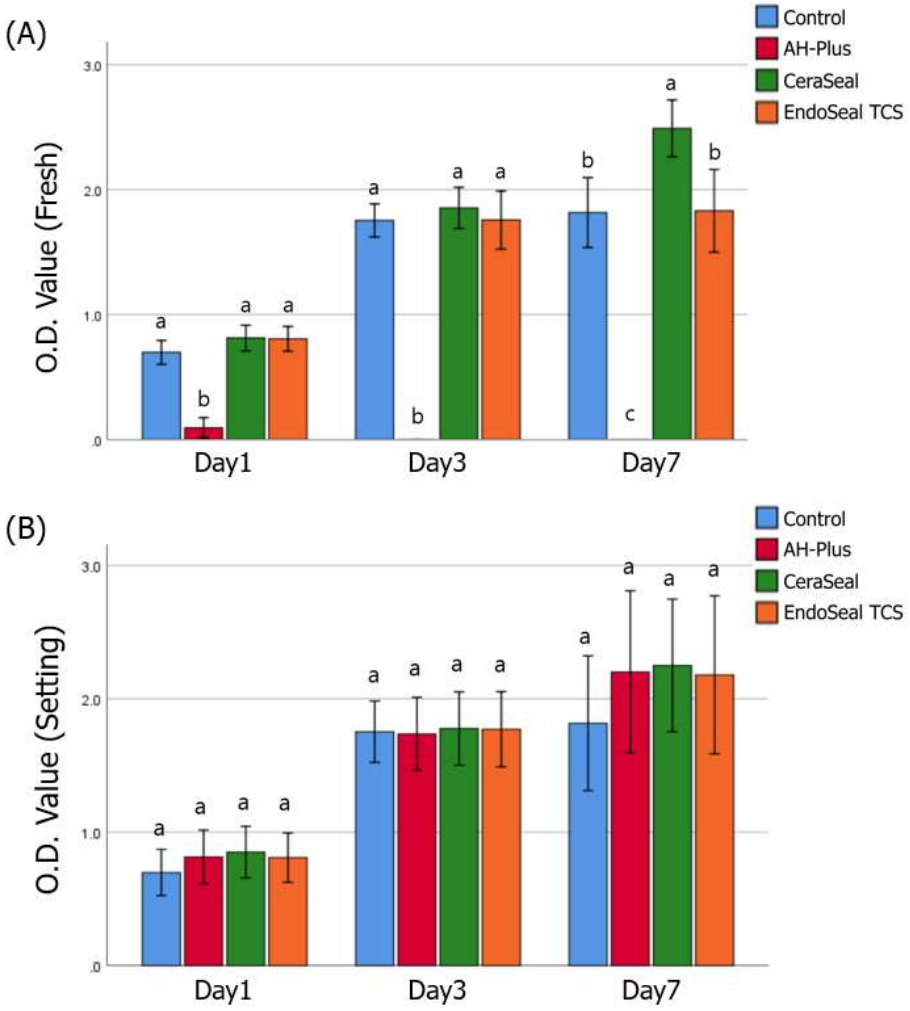

Fig 4. The effect of tested sealers on the cell viability of human periodontal ligament stem cells (hPDLSCs) using extraction media derived from (A) fresh and (B) set sealers. With the CCK-8 assay, absorbance values at 450nm were measured. Different alphabets indicate statistically significant differences in the same experimental time group ($p < 0.05$). Control group (normal hPDLSCs) was cultured in normal culture media with the same time as the experimental groups.

3. Evaluation of Inflammatory Response by ELISA

As shown in Figure 5, AH-Plus showed significantly higher expression of pro-inflammatory cytokines (IL-6 and IL-8) than other sealers, except on IL-6 in setting media (Figure 5B). Calcium silicate-based sealers remained statistically similar levels of IL-6 and IL-8 to control (cultured in normal culture media) (Figures 5A-D). In the anti-inflammatory cytokine TGF-β, all sealers in the fresh media were significantly lower than the control (Figure 5E). In the setting media, however, AH-Plus showed significantly lower expression of TGF-β than control and CeraSeal, and EndoSeal TCS was significantly lower than control. CeraSeal remained stastically similar levels to control (Figure 5F).

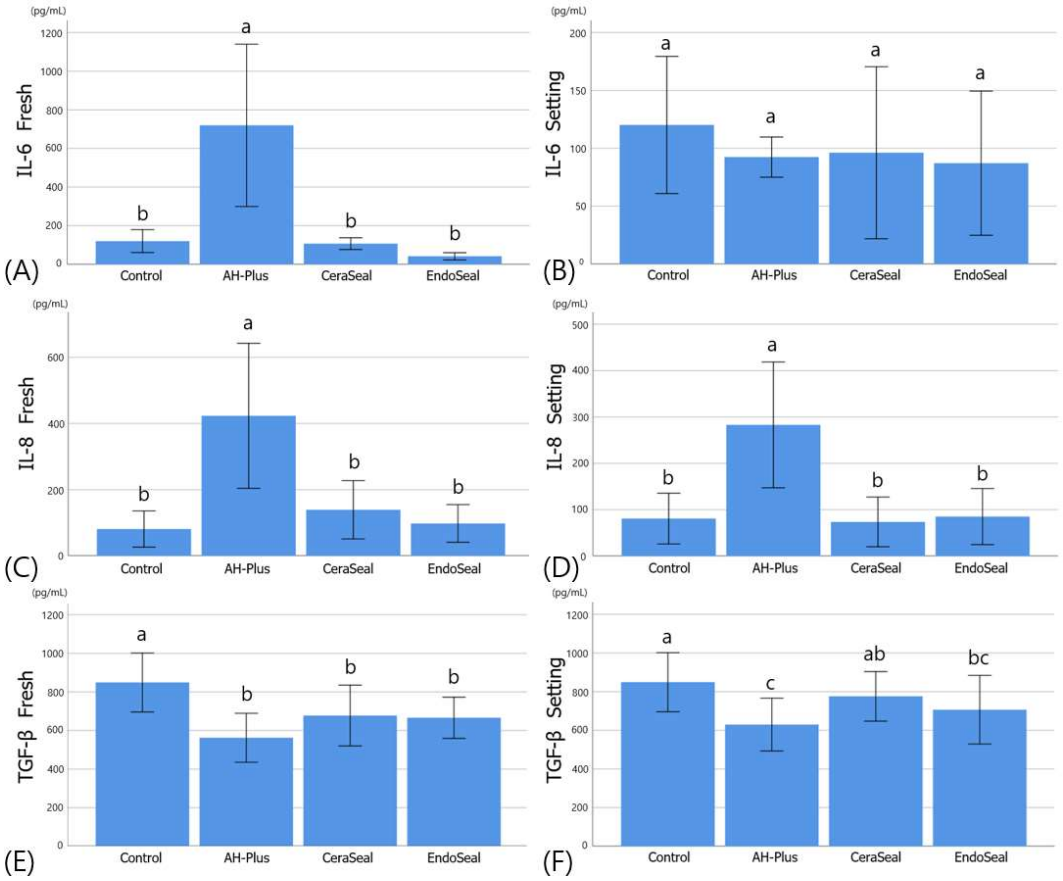

Fig 5. The effects of sealers on the inflammatory response of hPDLSCs. The expression of pro-inflammatory cytokines (IL-6 and IL-8) and anti-inflammatory cytokine (TGF-β) were measured using ELISA. (A) IL-6 expression in fresh media, (B) IL-6 expression in setting media, (C) IL-8 expression in fresh media, (D) IL-8 expression in setting media, (E) TGF-β expression in fresh media, (F) TGF-β expression in setting media. Different alphabets indicate statistically significant differences in the same experimental group, ($p < 0.05$). Control group (normal hPDLSCs) was cultured in normal culture media with the same time as the experimental groups.

4. Evaluation of Osteogenic potentials by RT-qPCR and staining

On day 3, all materials showed no significant difference in ALP, OCN, and RUNX2 expression (Figure 6). In ALP expression on day 7, AH-Plus was significantly lower than other materials (Figure 6A). In OCN expression on day 7, EndoSeal TCS was significantly higher than other materials, and AH-Plus was lower than control (cultured in osteoinduction media) (Figure 6B). In RUNX2 expression on day 7, EndoSeal TCS was significantly higher than the control, but there was no significant difference between the materials (Figure 6C).

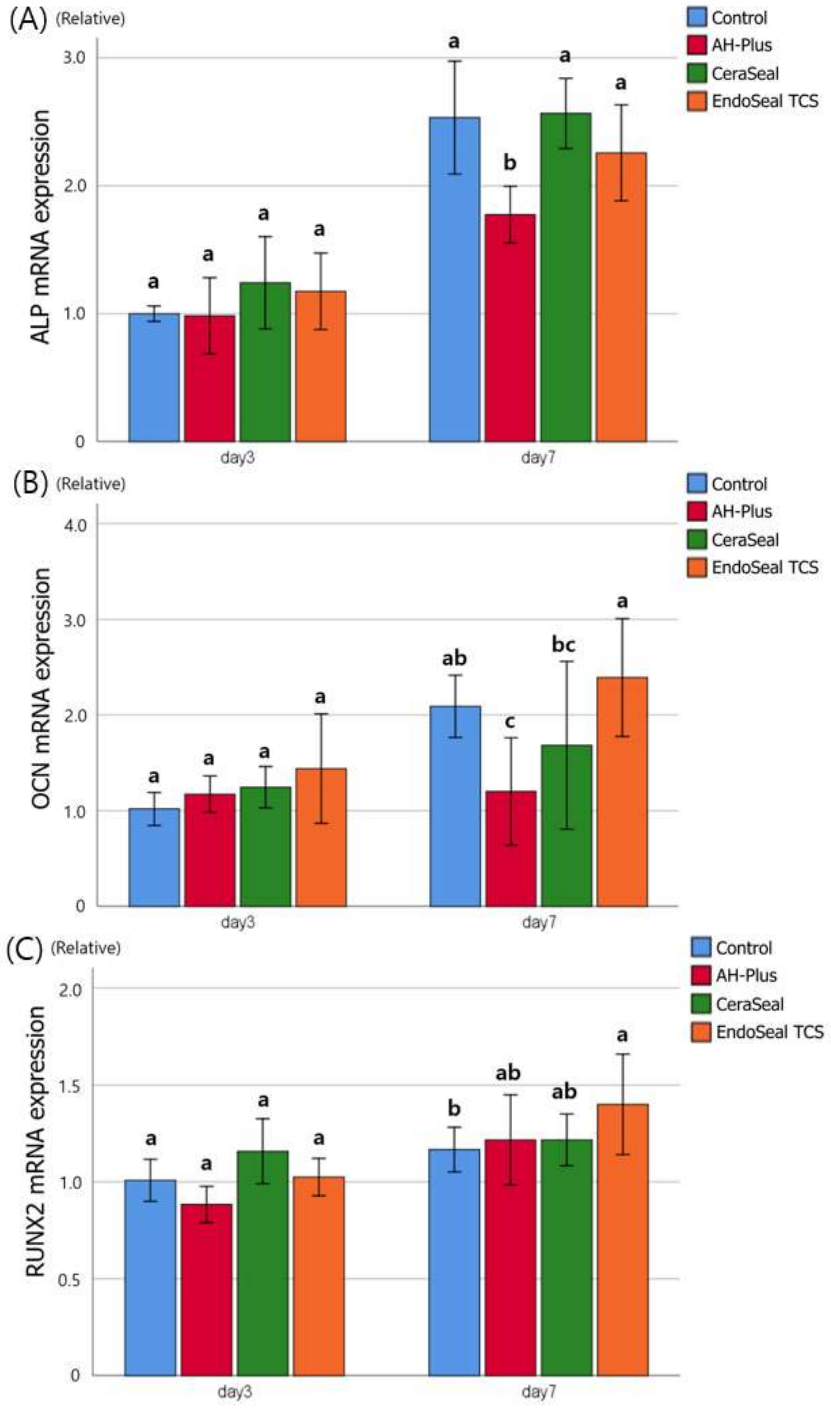

Fig 6. The effects of sealers of hPDLSCs on the relative mRNA expression of (A) ALP, (B) OCN, and (C) RUNX2 measured with RT-qPCR and normalized with β-actin. Different alphabets indicate statistically significant differences in the same experimental time group, ($p < 0.05$). Control group (osteoinducted hPDLSCs) was cultured in osteoinduction media with the same time as the experimental groups.

ALP staining (performed after 3days and 7days) and ARS staining (performed after 14days) are shown in Figure 7. In ALP staining on day 3, all materials are not stained enough to evaluate the difference. In ALP staining on day 7 and ARS staining on day 14, AH-Plus is less stained than calcium silicate-based sealers. CeraSeal and EndoSeal TCS showed similar ALP, ARS staining intensity to that of positive control (cultured in osteoinduction media).

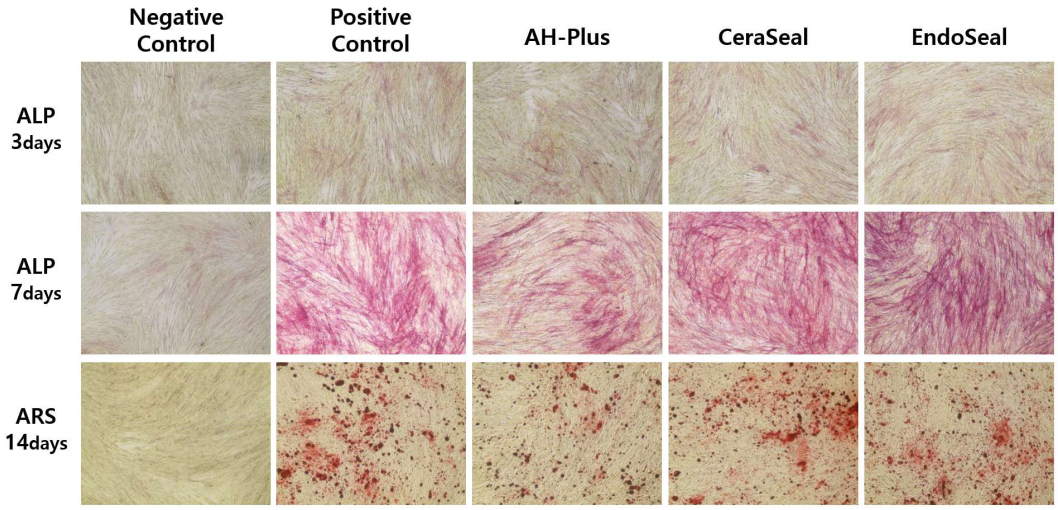

Fig 7. The effects of sealers on the osteogenic differentiation of hPDLSCs. ALP staining was performed after 3days, 7days, and ARS staining was done after 14days. 40X magnified microscope images are shown. Negative control, cultured in normal culture media; Positive control, cultured in osteoinduction media (normal culture media with osteogenic-inducing reagents); Experimental groups, cultured in setting media with osteogenic-inducing reagents

5. Evalulation of cell attachment and material surface morphology by Scanning Electronic Microscopy

Cell attachment of hPDLSCs on the set material disc and surface of set material disc itself were examined with secondary electron mode of scanning electron microscope (SEM) (Figure 8). No cell adhesion was observed on the set surface of AH-Plus, and several death cells and their debris were observed (Figure 8A). Whereas set surface of two calcium silicate set discs (CeraSeal and EndoSeal TCS) showed well-adhered hPDLSCs with production of extracellular matrix and high degree of cell proliferation (Figure 8C, 8E). By looking at the material surface next to the cells in CeraSeal, characteristic cubic particles can be identified (Figure 8C). The surface morphology of the materials was observed after 48 hours of material setting without 72 hours of additional cell incubation. AH-Plus showed resinous matrix and fine filler particles (Figure 8B). On the surfaces of CeraSeal and EndoSeal TCS, Honeycomb morphology or acicular spherule morphology is observed (Figures 8D and 8E).

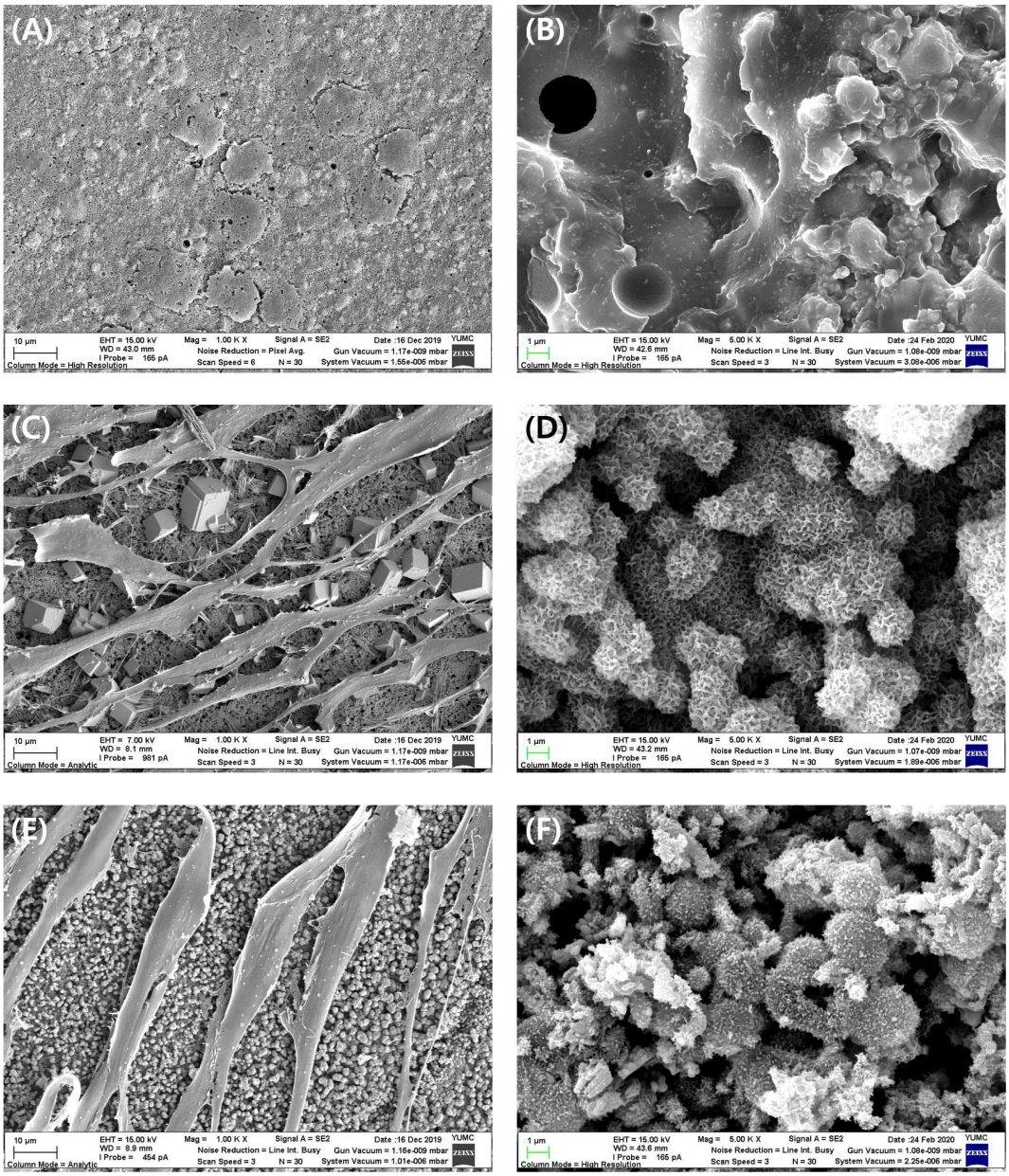

Fig 8. Cell attachment and morphology of hPDLSCs on the set (A) AH-Plus; (C) CeraSeal; and (E) EndoSeal TCS disc are shown at magnification of 1000X. After 48 hours of material disc setting, 72 hour of cell incubation was performed. Surface morphology of the

set (B) AH-Plus; (D) CeraSeal; and (F) EndoSeal TCS disc are shown at magnification of 5000X. 48 hours of material disc setting without cell incubation was performed.

IV. Discussion

In recent studies, epoxy resin-based sealers showed various tissue responses and cytotoxicity (Candeiro et al., 2016; Troiano et al., 2018). On the contrary, the newly introduced calcium silicate-based sealers are known to have biocompatibility, such as osteoconductivity and anti-inflammatory activity (Donnermeyer et al., 2018). However, less research has been done on calcium silicate based sealers than epoxy resin-based sealers. In this study, the biocompatibility of two recently developed calcium silicate-based sealers (CeraSeal, EndoSeal TCS) was evaluated, and they were found to be less cytotoxic and more biocompatible than epoxy resin-based sealer.

In this study, each experiment was conducted in fresh media or in setting media. Experiments in fresh media were conducted to evaluate the initial response by the unset sealer immediately after root canal filling. In fresh media, cell viability test and inflammatory response were measured, and the initial cell toxicity and initial inflammatory response after root canal filling were evaluated. Experiments in setting media were conducted to evaluate the long term response of the fully set sealer. In the setting media, mesenchymal phenotype measurement, cell viability test, inflammatory response measurement, and osteogenic potential evaluation were performed. Through this, long term responses of each aspect were evaluated.

hPDLSCs are a subpopulation of multipotent mesenchymal stem cells (MSCs) and they

have been used frequently in in-vitro biocompatibility studies (Zhu and Liang, 2015). In clinical situation, hPDLSCs may contact directly with extruded sealers, so these cells are good models for biocompatibility studies of root canal sealers. We incubated hPDLSCs in setting media for 3 and 7 days to see how each set sealers affects the stemness of hPDLSCs. The stemness of cultured cells was evaluated by MSC criteria (Dominici et al., 2006), expression levels of mesenchymal stem cell markers (CD90, CD105, and CD73) and the hematopoietic markers (CD11b, CD19, CD34, CD45, and HLA-DR) were measured using flow cytometry. In result, mesenchymal stem cell markers (CD90, CD105, and CD73) showed high level (> 99%) and the hematopoietic markers (CD11b, CD19, CD34, CD45, and HLA-DR) showed low expression level (<1%) in both Calcium silicate-based sealers and AH-Plus. This met the minimal criteria for defining MSCs (Dominici et al., 2006): mesenchymal stem cell markers expression level ≥ 95% and hematopoietic markers expression level ≤ 2%. Experimental groups showed similar expression levels compared to normal hPDLSCs acting as a positive control, therefore it can be confirmed that the stemness of hPDLSCs was well maintained in set medium of calcium silicate-based sealers and even AH-Plus.

Cell viability test (CCK-8 assay) and inflammatory response test (ELISA) were performed separately in fresh media and setting media. When the sealer is injected into the root canal, the fresh, unset sealer reacts with the periapical tissue. At this time, viability or immune response of the cell contacting the sealers is directly related to post-op pain and

initial toxicity (Lee et al., 2019). In fresh media, AH-Plus shows very high cytotoxicity at all time periods. The unset AH-Plus showed severe initial cytotoxicity in previous studies (Candeiro et al., 2016; Lee et al., 2019; Troiano et al., 2018), which is thought to be due to the toxicity of the epoxy resin, one of the major components of AH-Plus (Cohen and Hargraves, 2010). Calcium silicate-based sealers showed similar cell viability to control (cultured in normal culture media) at all time periods in fresh media. Notably, cell viability of CeraSeal significantly increased at day 7 than other groups include control. These results suggest that calcium silicate-based sealers have good biocompatibility and even in some cases have the potential to promote cell growth.

Cell viability in setting media was not significantly different between AH-Plus, CeraSeal, EndoSeal TCS and control in all experimental periods. AH-Plus, which showed high toxicity in fresh media, showed similar cell viability to control after fully set. This results was consistent with the previous study of AH-Plus, which shows initial toxicity but becomes well tolerated within a few weeks (Tomson et al., 2014). Calcium silicate-based sealers were confirmed to have low cytotoxicity even after completely set.

The inflammatory response of hPDLSCs to the material was evaluated using ELISA, and target cytokines were IL-6, IL-8, and TGF-β. IL-6 and IL-8 are pro-inflammatory cytokines that up-regulate the inflammatory response and can be associated with inflammatory responses such as post-op pain after canal obturation (Seong et al., 2016). AH-Plus showed significantly higher cytokine level than calcium silicate-based sealers in

IL-6 fresh group, IL-8 fresh and setting group, which is related to AH-Plus showing high toxicity in cell viability test (Diomede et al., 2014). The pro-inflammatory cytokine levels of the two calcium silicate-based sealers was not different from the control (cultured in normal culture media), and this result also represents one aspect of their excellent biocompatibility. TGF-β is an anti-inflammatory cytokine, and its presence is known to contribute to the healing of apical periodontitis (Dessaune Neto et al., 2018). In the fresh media, all the experimental groups showed lower levels of TGF-β than the control in the fresh media. In setting media, AH-Plus and EndoSeal TCS showed lower levels of TGF-β than control. However, CeraSeal showed higher level of TGF-β than AH-Plus, and there was no significant difference between CeraSeal and control. Through these results, it is possible to predict the anti-inflammatory effect of CeraSeal.

The osteogenic potential of hPDLSCs for the materials was quantified with RT-qPCR for ALP, OCN, and RUNX2, and visually qualified with ALP staining and ARS staining. If the sealers have osteogenic potential or at least do not inhibit osteogenic differentiation of hPDLSCs, apical healing can be promoted after canal obturation or even extrusion of the sealer may not interfere with the bony healing. ALP, OCN, and RUNX2 are essential transcription factors for osteogenic differentiation of mesenchymal stem cells (Mori et al., 2010). Among them, ALP is a factor involved in early stage of osteogenic differentiation, and OCN is a factor involved in late stage of osteogenic differentiation (Siew Ching et al., 2017). In addition, RUNX2 is a factor that regulates the overall process of osteogenic

differentiation (Bruderer et al., 2014). The more each of these factors is expressed, the more actively each process of osteogenic differentiation occurs (Bruderer et al., 2014; Siew Ching et al., 2017). From the results of day 7 of ALP, an early osteogenic differentiation marker, AH-Plus showed significantly lower level of the ALP expression than others. Other calcium silicate-based sealers were not significantly different from control (cultured in osteoinduction media). From the results of day 7 of OCN, a late osteogenic differentiation marker, EndoSeal TCS showed significantly higher expression of OCN than AH-Plus and CeraSeal. AH-Plus showed significantly lower level of OCN than control as in ALP. From the results of day 7 of RUNX2, there was no significant difference between the materials, but the EndoSeal TCS showed a significantly higher RUNX2 level than the control. EndoSeal TCS showed higher OCN levels than AH-Plus and CeraSeal, which may be related to the components of EndoSeal TCS. Both CeraSeal and EndoSeal TCS are based on calcium silicates. Among calcium silicates, CeraSeal is composed of a mixture of tricalcium silicates and dicalcium silicates, but EndoSeal TCS is composed only of tricalcium silicates. Tricalcium silicate is known to produce three times the amount of calcium hydroxide per molecule compared to dicalcium silicate (Bhatty and Bhatty, 1991; Camilleri, 2007). It is thought that EndoSeal TCS produces more calcium hydroxide than other materials, thereby showing a higher osteogenic potential (Chen et al., 2016). Likewise, AH-Plus is thought to have poor osteogenic potential because it does not have this action.

The results of ALP & ARS staining are similar to those of RT-qPCR. The ALP staining

kit reacts with ALP to make a purple product, and the ARS staining kit stains calcium deposits in red, allowing direct observation of calcium mineralization. In the ALP 7days and ARS 14 days groups, CeraSeal and EndoSeal TCS showed similar staining to positive control (cultured in osteoinduction media), whereas AH-Plus stained less. Based on the results of the ALP, OCN, RUNX2 mRNA expression test and ALP, ARS staining test, it is thought that EndoSeal TCS can exhibit better osteogenic potential than other materials and further promote bony healing. CeraSeal shows a similar value to control (cultured in osteoinduction media), so it is thought not to interfere with normal bony healing. On the other hand, AH-Plus is thought to inhibit bony healing, so care should be taken to ensure that AH-Plus is not extruded out of the apical foramen.

Cell attachment and morphology of hPDLSCs on the set material disc were examined with secondary electron mode of SEM. Since the additional process of culturing the cell itself may affect the crystal structure of the material (Camilleri et al., 2005), the surface structure was observed after 48 hours of material setting without cell culture. When the cells were cultured on the AH-Plus, an epoxy-resin based sealer, all cells were observed to be dead in a round shape that hardly differentiated (Figure 8A). This is the result corresponding to that AH-Plus showed high cell toxicity in the cell viability test of this study. Looking at the surface of the AH-Plus material (Figure 8B), resinous matrix is on the ground (Left side), and filler particles (Right side) that are characteristic of the AH-Plus can be observed in it (Huffman et al., 2009). Unlike the epoxy resin sealer, cells on the

surface of calcium silicate-based sealers are proliferated well and differentiated well. Looking at the cell growth on the CeraSeal surface (Figure 8C), hPDLSCs are well-adhered to the surface of the material and appear to be well differentiated, extending in all directions. On the cell surface, extracellular matrices with globular and reticulum form are also observed. Looking at the surface of the CeraSeal set for 48 hours (Figure 8D), the honeycomb appearance or acicular spherule appearance, which is a typical crystal form of calcium silicate hydrate (C-S-H), is observed (Berzins, 2014; Formosa et al., 2012). Also, looking at the surface of CeraSeal with cells, characteristic cubic forms are seen next to the cells. In the form of its crystals, it appears to be a typical form of C3AH6 ($3CaO{\cdot}Al_2O_3{\cdot}6H_2O$, Hydrogarnet) (Berzins, 2014; Hasegawa et al., 2018). C3A ($Ca_3(Al_2O_3)_2$, tricalcium aluminate), one of the constituents of CeraSeal, usually reacts with gypsum ($CaSO_4$, Calcium sulfate) in usual MTA to form needle-like ettringite(calcium sulfoaluminate). However, there are no such gypsum components in CeraSeal, so it is thought that cubic shaped C3AH6 was formed (Berzins, 2014). This C3AH6 phase is known to increase the initial strength of MTA (Berzins, 2014), and it is thought that the cubic crystals grew by hydration for an additional 72 hours during cell culture. The cell growth on the EndoSeal TCS disc was active like CeraSeal, and the cells were well differentiated into characteristic elongated forms (Figure 8E). Since the cells directly contacting calcium silicate-based sealers grew much better than epoxy-resin based sealer, it was confirmed that biocompatibility of calcium silicate-based sealers in cell level was

superior. The crystal form of EndoSeal TCS is observed with a typical honeycomb appearance or acicular spherule appearance. Through this, it is considered that the initial crystalline phase of EndoSeal TCS is calcium silicate hydrate (C-S-H) same as CeraSeal.

V. Conclusions

According to this study, it was confirmed that calcium silicate-based sealers (CeraSeal, EndoSeal TCS) are more biocompatible and less cytotoxic than epoxy-resin based sealer. In particular, CeraSeal showed less cytotoxicity than other materials before setting, and EndoSeal TCS showed better osteogenic potential than other materials.

References

Berzins DW (2014). Chemical properties of MTA. Mineral Trioxide Aggregate. Properties and clinical applications. Ames: Wiley Blackwell: 17-35.

Bhatty, Bhatty JI (1991). A review of the application of thermal analysis to cement-admixture systems. Thermochimica acta 189(2): 313-350.

Bruderer M, Richards R, Alini M, Stoddart MJ (2014). Role and regulation of RUNX2 in osteogenesis. Eur Cell Mater 28(28): 269-286.

Camilleri J (2007). Hydration mechanisms of mineral trioxide aggregate. International endodontic journal 40(6): 462-470.

Camilleri J, Montesin FE, Brady K, Sweeney R, Curtis RV, Ford TRP (2005). The constitution of mineral trioxide aggregate. Dental Materials 21(4): 297-303.

Candeiro GTdM, Moura-Netto Cd, D'Almeida-Couto R, Azambuja-Júnior N, Marques MM, Cai S, et al. (2016). Cytotoxicity, genotoxicity and antibacterial effectiveness of a bioceramic endodontic sealer. International endodontic journal 49(9): 858-864.

Chen L, Zheng L, Jiang J, Gui J, Zhang L, Huang Y, et al. (2016). Calcium Hydroxide–induced Proliferation, Migration, Osteogenic Differentiation, and Mineralization via the Mitogen-activated Protein Kinase Pathway in Human Dental Pulp Stem Cells. Journal of endodontics 42(9): 1355-1361.

Cohen S, Hargraves K (2010). Obturation of the cleaned and shaped root canal system. Pathway of the pulp 9: 367.

Collado-González M, Tomás-Catalá CJ, Oñate-Sánchez RE, Moraleda JM, Rodríguez-Lozano FJ (2017). Cytotoxicity of GuttaFlow Bioseal, GuttaFlow2, MTA Fillapex, and AH Plus on human periodontal ligament stem cells. Journal of endodontics 43(5): 816-822.

Diomede F, Caputi S, Merciaro I, Frisone S, D'Arcangelo C, Piattelli A, et al. (2014). Pro-inflammatory cytokine release and cell growth inhibition in primary human oral cells after exposure to endodontic sealer. International Endodontic Journal 47(9): 864-872.

Dessaune Neto N, Porpino MTM, Antunes HdS, Rodrigues RCV, Perez AR, Pires FR, et al. (2018). Pro-inflammatory and anti-inflammatory cytokine expression in post-treatment apical periodontitis. Journal of Applied Oral Science 26.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4): 315-317.

Donnermeyer D, Bürklein S, Dammaschke T, Schäfer E (2018). Endodontic sealers based on calcium silicates: a systematic review. Odontology: 1-16.

Formosa L, Mallia B, Bull T, Camilleri J (2012). The microstructure and surface

morphology of radiopaque tricalcium silicate cement exposed to different curing conditions. Dental Materials 28(5): 584-595.

Hasegawa G, Moriya S, Inada M, Kitano M, Okunaka M, Yamamoto T, et al. (2018). Topotactic Synthesis of Mesoporous 12CaO• 7Al2O3 Mesocrystalline Microcubes toward Catalytic Ammonia Synthesis. Chemistry of Materials 30(14): 4498-4502.

Huffman B, Mai S, Pinna L, Weller R, Primus C, Gutmann J, et al. (2009). Dislocation resistance of ProRoot Endo Sealer, a calcium silicate-based root canal sealer, from radicular dentine. International Endodontic Journal 42(1): 34-46.

Lee JK, Kim S, Lee S, Kim H-C, Kim E (2019). In vitro comparison of biocompatibility of calcium silicate-based root canal sealers. Materials 12(15): 2411.

Mori G, Centonze M, Brunetti G, Ballini A, Oranger A, Mori C, et al. (2010). Osteogenic properties of human dental pulp stem cells. Journal of Biological Regulators & Homeostatic Agents 24(2): 167-175.

Pascon EA, Spngberg LS (1990). In vitro cytotoxicity of root canal filling materials: 1. Gutta-percha. Journal of endodontics 16(9): 429-433.

Scarparo RK, Grecca FS, Fachin EVF (2009). Analysis of tissue reactions to methacrylate resin-based, epoxy resin-based, and zinc oxide–eugenol endodontic sealers. Journal of endodontics 35(2): 229-232.

Seong S, Kim JH, Kim N (2016). Pro-inflammatory cytokines modulating osteoclast

differentiation and function. Journal of Rheumatic Diseases 23(3): 148-153.

Siew Ching H, Luddin N, Ab Rahman I, Thirumulu Ponnuraj K (2017). Expression of odontogenic and osteogenic markers in DPSCs and SHED: a review. Current stem cell research & therapy 12(1): 71-79.

Tomson RM, Polycarpou N, Tomson P (2014). Contemporary obturation of the root canal system. British dental journal 216(6): 315.

Troiano G, Perrone D, Dioguardi M, Buonavoglia A, Ardito F, MUZIO LL (2018). In vitro evaluation of the cytotoxic activity of three epoxy resin-based endodontic sealers. Dental materials journal 37(3): 374-378.

Victoria-Escandell A, Ibañez-Cabellos JS, de Cutanda SB-S, Berenguer-Pascual E, Beltrán-García J, García-López E, et al. (2017). Cellular responses in human dental pulp stem cells treated with three endodontic materials. Stem cells international 2017.

Zhu W, Liang M (2015). Periodontal ligament stem cells: current status, concerns, and future prospects. Stem cells international 2015.

Abstract (In Korean)

인간 치주인대 줄기세포에 대한 최신의 칼슘 실리케이트 실러와 에폭시 레진 계 실러의 생체 적합성에 대한 비교 연구

오 한 슬

연세대학교 대학원

치의학과

(지도교수 김 의 성)

본 연구의 목적은 인간 치주인대 줄기세포에 대해 최신의 칼슘 실리케이트 실러 (CeraSeal 및 EndoSeal TCS)와 에폭시 레진 계 실러 (AH-Plus)의 다양한 측면에서의 생체적합성을 평가, 비교하기 위함이다.

인간 치주인대 줄기세포는 16세 ~ 24세 사이의 인간의 소구치 (n = 4)에서

획득되어 배양되었다. Fresh media를 만들기 위해 경화되지 않은 실러를 DMEM에 섞은 후 24시간 보관하였고, Setting media를 만들 때에는 실러를 작은 원판 형태로 48시간 경화시킨 후 DMEM에 24시간 동안 추출하였다. 각 실험 조건에 따라 세포들은 Fresh media 혹은 Setting media에서 배양되었다. 중간엽 줄기세포 표현형 검사는 세포를 Setting media에서 배양 후 CD11b, CD19, CD34, CD45, CD73, CD90, CD105, 및 HLA-DR 의 발현을 Flow Cytometry (FACS)를 이용하여 확인하였다. 세포 생존능 검사는 세포를 Fresh media 및 Setting media에 배양 후 CCK-8 키트를 사용하여 시행되었다. 재료에 대한 염증 반응을 평가하기 위해, Fresh media 및 Setting media에서의 IL-6, IL-8 및 TGF-β농도를 ELISA 키트를 이용하여 분석하였다. Setting media에서의 인간 치주인대 줄기세포의 골형성 능력은 ALP, OCN 및 RUNX2에 대한 RT-qPCR 검사로 수치화 하였고, ALP 염색과 ARS 염색으로 시각화하였다. 재료에 대한 세포 부착 및 성장, 그리고 재료 자체의 표면 특성은 주사 전자 현미경을 이용하여 확인하였다. 통계학적 유의차는 SPSS를 이용한 분산 분석과 Tukey's test로 확인하였다.

FACS 분석에서 모든 재료에서 중간엽 줄기세포 표지자는 높은 수준(>99%)를 보였고, 조혈세포 표지자는 낮은 수준(<1%)을 보였다. 따라서 모든 재료가 인간 치주인대 줄기세포의 줄기세포능을 해치지 않는 것을 확인하였다. Fresh media에서 시행한 세포 생존능 실험에서, AH-Plus는 모든 실험

기간 동안 가장 낮은 세포 생존능을 보였으며, CeraSeal은 다른 군들 보다 통계학적으로 유의미하게 더 좋은 세포 생존능을 나타내었다. Setting media에서는 모든 실험 기간에서 재료 사이에 통계학적 유의차가 없었다. ELISA 실험에서 AH-Plus는 염증 촉진 사이토카인인 IL-6, IL-8의 발현이 다른 재료들에 비해 유의차있게 높았다. 항염 사이토카인인 TGF-β에서는 CeraSeal만 양성대조군과 비슷한 수준을 보였고, AH-Plus와 EndoSeal TCS는 더 낮은 TGF-β수준을 보였다. 골 형성 표지자에 대한 RT-qPCR 실험에서는, AH-Plus는 전반적으로 칼슘 실리케이트 실러에 비해 낮은 수준을 보였고, EndoSeal TCS는 다른 군들에 비해 더 높은 수준을 보였다. ALP, ARS 염색 결과 칼슘 실리케이트 실러는 양성 대조군과 비슷한 정도로 염색이 되었지만, AH-Plus는 그보다 덜 염색이 되었다. 마지막으로, 주사 전자 현미경을 이용하여 세포 성장과 부착을 확인하였을 때 AH-Plus는 세포가 모두 죽어있었으며 거의 분화하지 못한 형태였다. 반면에 칼슘 실리케이트 실러에서는 세포가 잘 성장하였으며, 높은 수준으로 분화한 것을 확인하였다.

결론적으로, 칼슘 실리케이트 실러 (CeraSeal 및 EndoSeal TCS)는 기존의 에폭시 레진 계 실러인 AH-Plus보다 생체적합성이 더 뛰어난 것을 확인하였다. 특히 CeraSeal은 경화 전 다른 재료들보다 세포 독성이 더 적었고, EndoSeal TCS는 다른 재료들보다 더 골형성 능력이 뛰어났다.

핵심 되는 말 : 생체적합성; 근관 충전 실러; 칼슘 실리케이트; 에폭시 레진; 세포 생존능; 골형성 분화능; 주사전자현미경