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RESEARCH ARTICLE

Biocompatibility and Bioactivity of Four Different Root Canal Sealers in Osteoblastic Cell Line MC3T3-El

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Background: Endodontic sealers or their toxic components may become inflamed and lead to delayed wound healing when in direct contact with periapical tissues over an extended period. Moreover, an overfilled sealer can directly interact with adjacent tissues and may cause immediate necrosis or further resorption. Therefore, the treatment outcome conceivably depends on the endodontic sealer's biocompatibility and osteogenic potential. This study aimed to evaluate the cell viability and osteogenic effects of four different sealers in osteoblastic cells.

Methods: AH Plus (resin-based sealer), Pulp Canal Sealer EWT (zinc oxide-eugenol sealer), BioRoot RCS (calcium silicate-based sealer), and Well-Root ST (MTA-based calcium silicate sealer) were mixed strictly according to the manufacturer's instructions, and dilutions of sealer extracts (1/2, 1/5 and 1/10) were determined. Cell viability was measured using the water-soluble tetrazolium-8 (WST-8) assay. Differentiation was assessed by alkaline phosphatase (ALP) activity and mineralized nodule formation by Alizarin Red S staining.

Results: The cell viability of the extracts derived from the sealers excluding Well-Root ST was concentration dependent, with sealer extracts having the least viability at a 1/2 dilution. At sealer extract dilution of 1/10, the test groups showed the same survival rate as that control group, with the exception of BioRoot RCS. Among all experimental groups, BioRoot RCS showed the highest cell viability after 48 hours. The ALP activity was significantly higher in a concentration-dependent manner. Furthemore, all four materials promoted ALP activity and mineralized nodule formation compared to the control at 1/10 dilutions.

Conclusion: This is the first study to highlight the differences in biological activity of these four materials. These results suggest that the composition of root canal sealers appears to alter the form of biocompatibility and osteoblastic differentiation.

Key Words: Biocompatibility, Cell differentiation, Osteoblasts, Root canal filling materials

Introduction

Endodontic sealers conventionally used to fill root canals affect the prognosis of endodontic treatment[1]. Although endodontic sealants are designed to remain within root canals during endodontic treatment, they sometimes extrude through apical narrowing[2,3]. The anatomical structure of the apical foramen, lateral canals, and dentinal tubules may allow tissue fluids to easily penetrate the root canal system, leading to degradation of the sealing material and subsequent leaching of various components[4]. When it is in direct contact with the periapical tissue for a long time, endodontic sealers or their toxic components may become inflamed and delay wound healing[5]. Moreover, the overfilled sealer may interact with adjacent tissues, resulting in immediate necrosis or further resorption[6]. Even in the absence of extrusion, root canal sealer may release soluble toxic substances into the periapical tissues, affecting local bone metabolism[7]. Therefore, treatment outcomes conceivably depend on the endodontic sealer's biocompatibility and

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osteogenic potential.

Numerous types of root canal sealers are used in clinical settings, such as; AH Plus (resin-based sealer; Dentsply/De Trey, Konstanz, Germany), Pulp Canal Sealer EWT (zinc oxide-eugenol sealer; SybronEndo, Glendora, CA, USA), BioRoot RCS (calcium silicate-based sealer; Septodont, St. Maur-des-Fosses, France), and Well-Root ST (MTA-based calcium silicate sealer; Vericom, Chuncheon, Korea). AH Plus is a paste-to-paste material that exhibit an amine polymerization reaction contained in epoxy resin. It is a thermoplastic epoxy resin-based sealer with excellent physicochemical properties, despite a lack of bioactive potential[8]. Pulp Canal Sealer EWT is a zinc oxide eugenol sealer consisting of a powder base and liquid catalyst. Several studies have demonstrated, its toxic potential due to isolated eugenol released in mixing zinc oxide and eugenol[9,10]. However, studies show that Pulp Canal Sealer EWT produces better tissue organization than epoxy resin-based sealer after subcutaneous implantation in rat connective tissues[11]. BioRoot RCS is a bioactive mineral root canal sealant based on "active biosilicate technology," an innovative mineral micro-agglomerating chemical[12]. BioRoot RCS mainly consists of tricalcium silicate and zirconium oxide powder mixed with a liquid containing calcium chloride. This sealer is biocompatible and has a positive effect on biological activity and cell metabolism[13]. Well-Root ST, a white MTA calcium silicate sealer, incorporates bioactive glass. A previous study showed that, sealers containing MTA are highly biocompatible and stimulate mineralization[14]. They exhibit bioactivity by encouraging differentiation and migration of cells that produce hard tissue[15].

The biocompatibility and bioactivity of two newly developed calcium silicate-based sealers have not been fully elucidated. Therefore, this study aimed to evaluate the cell viability and osteogenic effects of four sealers on MC3T3-E1 cells which are osteoblastic cell lines, at different dilutions.

Materials and Methods

1. Sealer extract preparation

Four root canal sealers were evaluated (Table 1): AH Plus (resin-based sealer), Pulp Canal Sealer EWT (zinc oxide-eugenol sealer), BioRoot RCS (calcium silicate-based sealer), and Well-Root ST (MTA-based calcium silicate sealer). One spoon of BioRoot RCS powder was mixed with five drops of liquid, and the other sealers (AH Plus, EWT, Well-root ST) were mixed in a 1:1 ratio, according to the manufacturer's instructions. Disks of all root canal sealers were packed into Teflon molds (total sample weight: 2 g; size=6-mm diameter and 2-mm height) and stored at 37°C and 100% humidity for 24 hours to achieve complete setting. After setting, the eluates of the different materials were extracted under sterile conditions using α-minimal essential medium (α-MEM; Welgene, Gyeongsan, Korea). The ratio between the weight of the sample and volume of the culture medium was 0.05 g/mL for 72 hours at 37°C in a humid atmosphere containing 5% CO_2. The extraction media were collected at the end of this period and sterilized by passing them through a membrane filter (0.2 μm pore size; Sartorius, Göttingen, Germany). Subsequently, in pretest sealer concentrations, dilutions of sealer extracts (1/2, 1/5,

Table 1. Composition of Root Canal Sealers Evaluated in This Study

Root canal sealer	Composition
AH Plus (Dentsply/De Trey, Konstanz, Germany)	Paste A: bisphenol-A epoxy resin, bisphenol-F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: dibenzyldiamine, aminoadamantane, tricyclodecane-diamine, calcium tungstate, zirconium oxide, silica, silicone oil
Pulp Canal Sealer EWT (SybronEndo, Glendora, CA, USA)	Powder: silver powder, zinc oxide, thymol iodide, dimeric acid resin Liquid: clove oil, canada balsam
BioRoot RCS (Septodont, St. Maur-des-Fosses, France)	Powder: tricalcium silicate, zirconium oxide, povidone Liquid: aqueous solution of calcium chloride and olycarboxylate
Well-Root ST (Vericom, Chuncheon, Korea)	Calcium aluminosilicate compound, zirconium oxide, filler, thickening agent

and 1/10 dilutions) were determined.

2. Cell culture condition

The MC3T3-E1 mouse pre-osteoblast cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The MC3T3-E1 cells were cultured in α-MEM containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with an antibiotic-antimycotic solution (100 units/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml Fungizone® (amphotericin B); Gibco) at 37°C in a humidified atmosphere with 5% CO_2. The cell culture medium was replaced every 3 days.

3. Cell viability assay

The cell viability of various root canal sealers was measured using the water-soluble tetrazolium-8 (WST-8; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) assay (MediFab, Seoul, Korea). MC3T3-E1 cells were seeded in 96-well plates at an equal density of 1×10^4 cells per wells. After overnight incubation, the cells were cultured in the presence or absence (control) of sealer extracts (1/2, 1/5, and 1/10 dilutions) in a humidified atmosphere of 5% CO_2 at 37°C for 24 and 48 hours. The cells were incubated with WST-8 solution (5μL/well) for 2 hours. Then, absorbance at 450 nm was

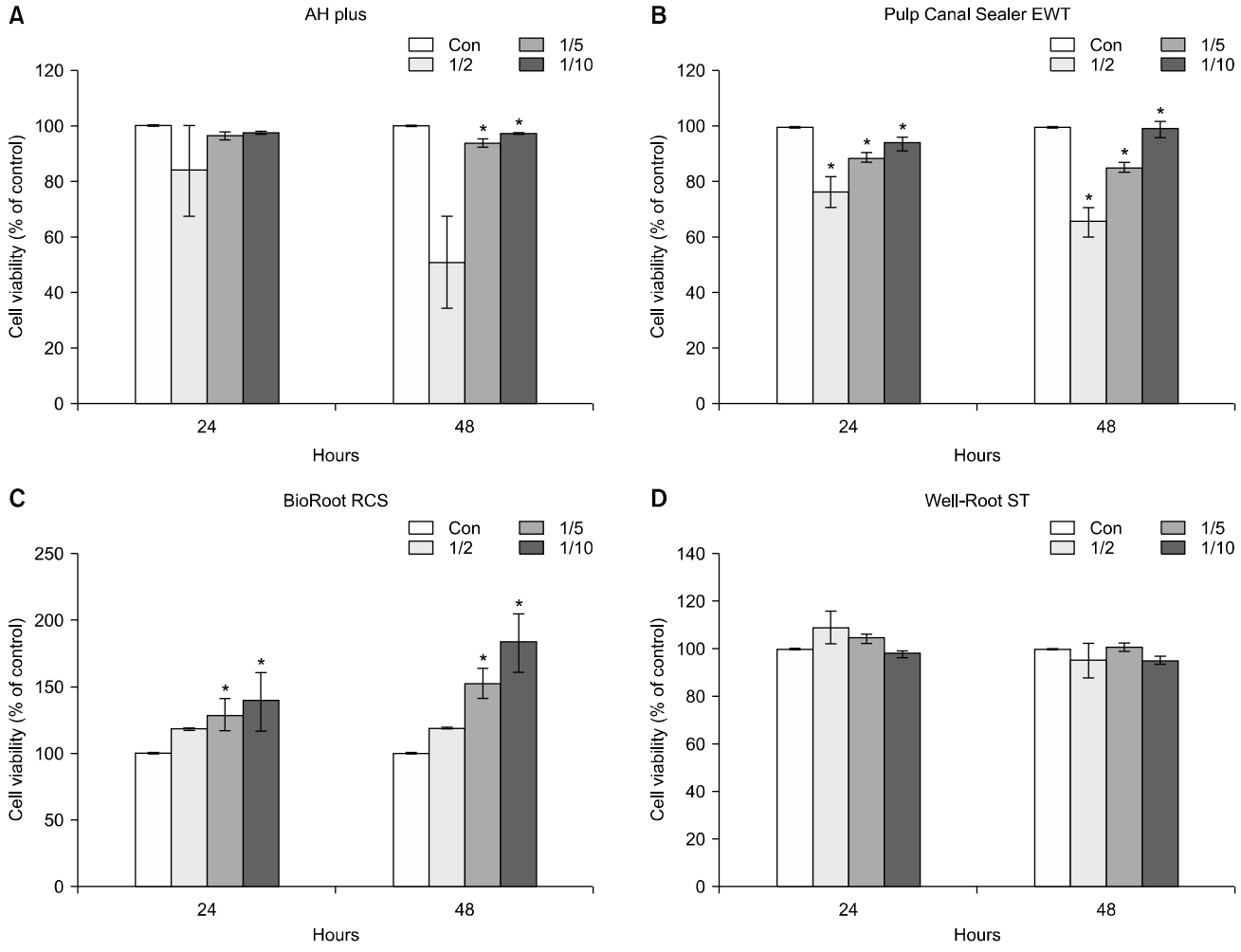

Fig. 1. Cell viability on various dilution of four root canal sealers. The percentage of cell viability was evaluated by the WST-8 assay using (A) AH Plus, (B) Pulp Canal Sealer EWT, (C) BioRoot RCS, and (D) Well-Root ST at 24 and 48 hours. MC3T3-E1 cells were cultured in extraction media derived from four sealers (1/2, 1/5, and 1/10 dilutions). Groups with letters above the data bar showed statistically significant results compared to the control group (n=3, *$p<0.05$). Con: control.

measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

4. Alkaline phosphatase (ALP) activity assay

MC3T3-E1 cells were cultured for 3, 5, and 10 days in osteogenic supplements (OS; α-MEM with 10% FBS with 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) at 1×10^4 cells per well in 96-well plates with sealer extracts (1/2, 1/5, and 1/10 dilutions). ALP activity was analyzed following the protocol recommended in the SensoLyte® pNPP ALP assay colorimetric kit (Anaspec, San Jose, CA, USA). Absorbance was measured spectrophotometrically at 405 nm using a microplate reader (Bio-Rad).

5. Mineralization assay

To quantify mineralization potential, MC3T3-E1 cells were cultured for 5 days in OS at 3×10^4 cells per well in a 24-well plate with sealer extracts (1/2, 1/5, and 1/10 dilutions). After 5 days, cells were stained with 2% Alizarin Red S (Sigma-Aldrich, St. Louis, MO, USA). At the end of the culture period, calcium deposits within cells and the extracellular matrix were visualized using bright-field microscopy (Olympus Corporation, Tokyo, Japan).

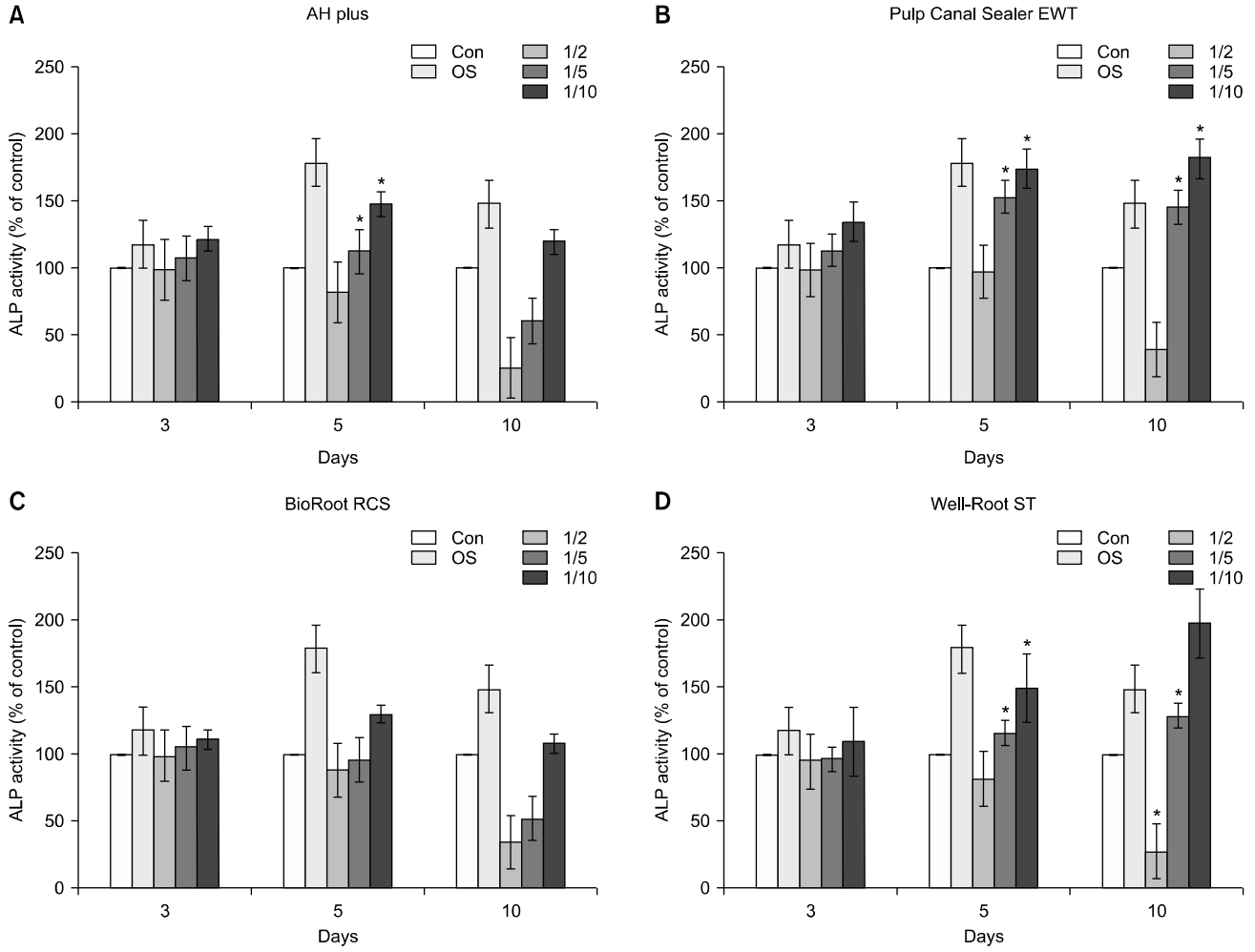

Fig. 2. Alkaline phosphatase (ALP) activity on various dilution of four root canal sealers. ALP activity demonstrated the osteogenic differentiation potential of the sealer extracts (A) AH Plus, (B) Pulp Canal Sealer EWT, (C) BioRoot RCS, and (D) Well-Root ST. The cells were incubated for 3, 5, and 10 days in sealer extracts (1/2, 1/5, and 1/10 dilutions) with odontogenic supplements (OS; α-MEM with 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate). Groups with letters above the data bar are statistically significant results compared to the control group (n=3, *$p<0.05$).

6. Statistical analysis

All data are presented as mean±standard deviation from a minimum of three replicates. The means of the treatment groups were compared to those of controls using the Student's t-test in SPSS Statistics 25 (IBM Corp., Armonk, NY, USA), and the statistical significance level was $p < 0.05$.

Results

1. Cytocompatibillity of MC3T3-E1 with four different root canal sealers

We determined the cell viability of each root canal sealer extract (various dilutions: 1/2, 1/5, and 1/10) (Fig. 1). At 1/10 dilution, AH26 Plus and Pulp Canal Sealer EWT showed nearly 100% cell viability in MC3T3-E1 cells up to until 48 hours but showed decreased cell viability at 1/2 dilution compared to the control (without sealer extracts). Among all experimental groups, BioRoot RCS showed the highest cell viability up to 48 hours ($p < 0.05$). Well-Root ST had similar cell viability compared to the controls without a cytotoxic effect at dilutions ranging from 1/2 to 1/10.

2. Osteogenic differentiation capacity of four different root canal sealers

The early differentiation of MC3T3-E1 osteoblasts was evaluated as a function of ALP activity. Data on ALP ratios (%) in the presence of different sealer extracts are shown in Fig. 2. At dilutions ranging from 1/5 to 1/10, sealers exhibited higher ALP activity than the control (without sealer extracts). In all experimental groups, the ALP index tended to increase with the lower sealer concentrations. At 1/10 dilution, Pulp Canal Sealer EWT and Well-Root ST exhibited significantly higher ALP activity than the control groups ($p < 0.05$).

Mineralization was assessed by Alizarin Red S staining. As expected, OS increased mineralized nodule formation in a time-dependent manner compared to the control. At 1/10 dilution, the formation of mineralized nodule formation was greater with Pulp Canal Sealer EWT and Well-Root ST, but not with other sealer extracts (Fig. 3).

Discussion

The importance of biological compatibility of root canal sealers is based on the fact that, during endodontic treatment, root canal sealers may exceed the root apex and penetrate periodontal tissues[16]. The tissue response to these materials may influence root canal treatment outcomes[17]. Biocompatible and bioactive root canal sealers can promote the reorganization of inflamed tissue and wound healing in apical periodontitis[18]. This study aimed to evaluate the biocompatibility and osteoblastic mineralization activity of various sealers.

In this study, the cell viability of four different sealers was analyzed using the WST-8 assay. AH Plus, a resin-based canal sealer, is known for its low bioactive potential[9]. This study confirmed the cytotoxic potential of AH Plus sealer and Pulp Canal Sealer EWT. In the case of AH Plus, despite the small amount of formaldehyde released, cytotoxicity is high because epoxy resin, one of

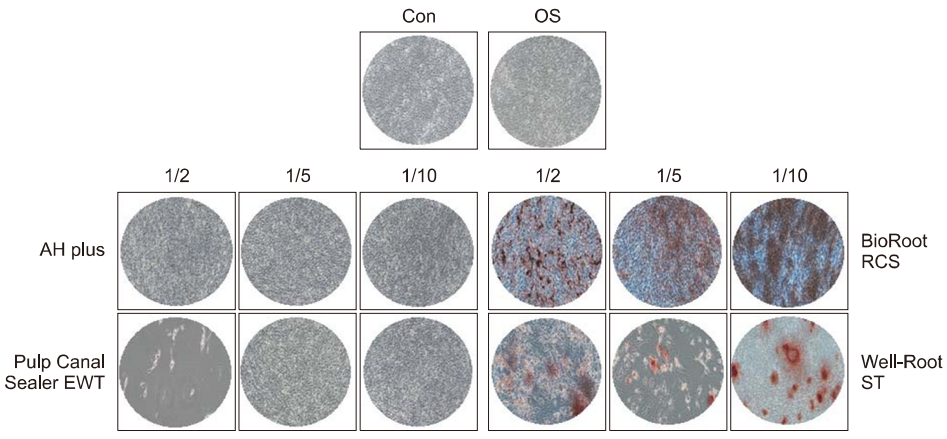

Fig. 3. Mineralization effects on various dilution of four root canal sealers. Mineralized nodule formation was assessed by staining with Alizarin Red S (light microscope, ×100). The cells were incubated in sealer extracts (1/2, 1/5, and 1/10 dilutions) with odontogenic supplements (OS; α-MEM with 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate) medium for 10 days. The data are representative of three independent experiments. Con: control.

the main components, has been identified as a toxic factor[19]. The epoxy resin present in AH Plus is a mutagen that may cause cell DNA strand breaks[20]. However, other studies have shown no significant periapical tissue damage even with extrusion of AH Plus sealer, implying low cytotoxic potential[21].

Zinc oxide eugenol type sealers, Pulp Canal Sealer EWT, are irritating mainly because of eugenol[10]. Nevertheless, Pulp Canal Sealer EWT, also known as a bioceramic sealer, has become increasingly popular owing to its ability to bond to dentin and form hydroxyapatite. Reports show that this sealer is biocompatible and stable in biological environments[9].

BioRoot RCS is a novel bioceramic endodontic sealer, with higher cell proliferation rates, than other sealers, such as epoxy resin-based and zinc oxide-eugenol sealers[13]. Our study showed that BioRoot RCS was the only sealer with good cell viability at all extract concentrations (Fig. 1).

Well-Root ST is a premixed, ready-to-use, injectable bioceramic cement paste developed for permanent obturation of the root canal. Well-Root, as described by the manufacturer, consists of zirconium oxide, calcium silicate, filler, and thickening agents. According to the results of this study, MTA calcium silicate-based sealers (Well-Root ST) appear to be more biocompatible and less cytotoxic than epoxy resin-based sealers. Well-Root ST, a bioceramic-based sealer composed of bioceramic powder and carrier, exhibits biocompatibility, biomineralization, and osteoconductivity[22,23].

Assessment of the activity of ALP, an enzyme expressed during the early maturation of osteoblasts, can determine the potential of inducing mineralized tissue formation[24]. Studies revealed that ALP activity in osteoblastic MC3T3-E1 cells[25] showed significant suppression with dental resin-based materials. Formaldehyde released from resin-based sealers can significantly decrease ALP activity in rats[26].

Alizarin Red S staining evaluates the mineralization activity of a substance by identifying calcium deposits in cell culture. The Alizarin Red S staining kit stains calcium deposits in red, allowing observation of calcium mineralization. The results showed that AH Plus and Pulp Canal Sealer EWT could inhibit bone healing. Therefore, care should be taken to prevent extrusion of AH Plus, and Pulp Canal Sealer EWT is not extruded out of the apical foramen[27].

This study highlights the significance of the first time that the four materials exhibit different biological activities. Within the limitations of this in vitro study, these results suggest that composition of root canal sealers can exert varying influences on biocompatibility and osteoblastic differentiation.

Notes

Conflict of interest

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

Ethical approval

This project does not require IRB review because it is an experimental paper using commercially available cells.

Author contributions

Conceptualization: Sang-Im Lee and Nu-Ri Jun. Data acquisition: Nu-Ri Jun. Formal analysis: Sang-Im Lee, Sun-Kyung Lee, and Nu-Ri Jun. Funding: Sang-Im Lee. Supervision: Sang-Im Lee. Writing–original draft: Sang-Im Lee, Sun-Kyung Lee, and Nu-Ri Jun. Writing–review & editing: Sang-Im Lee.

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