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Evaluation of Cytotoxicity and Osteogenic Potential of Strontium Silicate and Calcium Silicate-Based Sealers

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ABSTRACT

Objectives: This study aimed to evaluate the cytotoxicity and osteogenic potential of three sealers, including a strontium silicate-based sealer, C-Root SP (C-R), and two calcium silicate-based sealers, iRoot SP (i-R) and AH Plus Bioceramic Sealer (AHPbcs), compared with AH Plus (AHP) on MC3T3-E1 pre-osteoblasts.

Materials and Methods: Standardized sealer discs were eluted in a culture medium to assess cytotoxicity using the CCK-8 assay at various dilutions (1:1, 1:2, 1:5, and 1:10). Osteogenic differentiation was evaluated by culturing cells in osteogenic medium supplemented with 1:5 diluted sealer extract. Alkaline phosphatase (ALP) activity was assessed with an ALP assay and staining on days 7 and 14. Mineralized nodule formation was observed using Alizarin Red S staining on day 21. Gene expression of osteogenic markers (ALP, COL1A1, and RUNX2) was examined by RT–qPCR. Differences were analysed using one-way/two-way variance analysis with the Tukey post-hoc test. Statistical significance was established at $P < .05$.

Results: C-R, i-R, and AHPbcs showed significantly higher cell viability than AHP ($P < .001$). All sealers exhibited cytotoxicity at higher concentrations (1:1 and 1:2 dilutions). ALP activity was significantly lower in cells exposed to AHP compared to cells exposed to C-R, i-R, and AHPbcs ($P < .01$). Cells exposed to C-R, i-R, and AHPbcs exhibited higher mineralized nodule formation than cells exposed to AHP. C-R, i-R, and AHPbcs enhanced osteogenic differentiation with higher osteogenic gene expression than AHP ($P < .001$).

Conclusions: Within the limitations of the study, C-R, i-R, and AHPbcs were biocompatible with MC3T3-E1 pre-osteoblasts at lower concentrations and were able to enhance their osteogenic potentials.

Clinical relevance: The strontium silicate-based sealer shows a favourable biological response and osteogenic activity in vitro, comparable to calcium silicate-based sealers, indicating its potential for clinical applications.

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Introduction

Root canal instrumentation, disinfection, and obturation are essential steps for eliminating bacteria and preventing re-infection in the root canal system, enabling a successful endodontic outcome.[1] Root canal obturation at least offers two-fold effects: a fluid-tight seal to entomb residual pathogens and prevention of leakage from the oral cavity and periapical tissues to revive the residual pathogens.[2,3] It usually requires a core material and a root canal sealer as root filling to ensure a durable and impervious seal.[4] Root canal sealers may contact alveolar bone and periapical tissues directly or indirectly and release certain chemicals, causing various reactions in the periapical tissues.[5] Sealer extrusion beyond the apex or across lateral canals is possible, which can be irritative and may lead to inflammatory reactions.[6,7] They can also stimulate periapical epithelial proliferation, inducing foreign body reactions.[8] Therefore,

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overfilling can impair and delay periapical healing and may overwhelm the immunologic capability of the host, thereby lowering success.[2,9]

Biocompatibility is an essential characteristic of endodontic materials. Calcium silicate-based sealers have demonstrated excellent biocompatibility,[10] while epoxy resin sealers are most toxic when freshly mixed and become less toxic after being fully set.[11,12] With an improved understanding of bioceramics in enhancing biological response, root-filling materials have been modified to improve periapical healing. Specifically, the substitution of calcium with strontium has been suggested in calcium silicate-based materials, driven by the increasing use of strontium-substituted bioactive glass in orthopaedic surgery, where strontium ions have been shown to enhance bone regeneration by upregulating osteoblastic differentiation and inhibiting bone resorption by osteoclasts.[13] C-Root SP was introduced in 2021 as the first commercial strontium silicate-based sealer and has been demonstrated to be biocompatible with human periodontal ligament stem cells and to induce osteogenic differentiation. However, its bioactivity with other cell types has not been explored, and evidence regarding strontium substitution in endodontic sealers is scarce. Since periapical healing depends on osteoblasts, which secrete collagen and growth factors to allow osteoid formation that mineralizes to form bone,[14] the interaction between the endodontic sealer and osteoblasts in the periapical area is crucial in regulating apical lesion healing processes. Research on this novel strontium silicate-based sealer is scarce, and its effects on osteoblastic activity remain uncertain. The study aimed to evaluate the in vitro effects of three sealers, ie, C-Root SP, iRoot SP, and AH Plus Bioceramic Sealer, on the cell viability and osteogenic potential of MC3T3-E1 pre-osteoblasts compared with AH Plus.

Materials and methods

Cell culture

MC3T3-E1 pre-osteoblasts (ATCC, CRL-2594; Manassas, VA, USA) were cultured in alpha-minimum essential medium (α-MEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin–streptomycin (Thermo Fisher Scientific), containing 100 U/mL penicillin and 100 mg/mL streptomycin, at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of extraction medium

Four root canal sealers were evaluated: C-Root SP (C-R), iRoot SP (i-R), AH Plus Bioceramic Sealer (AHPbcs), and AH Plus (AHP) (Table 1). C-R, i-R, and AHPbcs were commercially available in a pre-mixed state and prepared using injectable syringes, while AHP was mixed according to the manufacturer's instructions. To fabricate sealer discs, sealers were injected into sterilized silicone moulds with a thickness of 5 mm and an internal diameter of 6 mm, and covered with Mylar sheets on both surfaces under aseptic conditions. Samples were allowed to set for 72 hours at 37°C in a humidified 5% CO_2, 95% air atmosphere. After setting, the sealer discs were sterilized by ultraviolet radiation for 1 hour on each surface. They were then immersed in culture medium to obtain an extraction ratio of 3 cm^2/ml according to ISO10993-12 standards and incubated in a humidified 5% CO_2, 95% air atmosphere at 37°C for an additional 72 hours. The suspension was centrifuged at 1500 g for 5 minutes, and the supernatant was filtered through a sterile 0.22 μm filter (Sigma-Aldrich, St. Louis, MO, USA). The collected extract was serially diluted with medium to 1:2, 1:5, and 1:10 for the experiments, based on the dilutions used in previous studies.[15,16]

Cell viability assay

To assess the cytotoxicity of the sealers, cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assays (Dojindo, Kumamoto, Japan). MC3T3-E1 pre-osteoblasts were seeded in 96-well plates (Corning Corp, Corning, NY, USA) at an initial density of 5×10^3 cells per well. After 1 day, the medium was changed to different dilutions of extracts (1:1, 1:2, 1:5, and 1:10). Controls were cultured without test sealers. The growth medium was changed every 3 days. After 3 and 7 days of cell exposure to sealer extracts (or non-exposure in the control group), cell viability was assessed using CCK-8 assays (Dojindo) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (SpectraMax i3, Molecular Devices, San Jose, CA, USA). Cell viability of MC3T3-E1 pre-osteoblasts was calculated as a

Table 1 – Endodontic sealers used in the study and their chemical composition.

Material	Manufacturer	Composition
AH Plus (AHP)	Dentsply DeTrey GmbH, 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.
AH Plus Bioceramic Sealer (AHPbsc)	Dentsply, Tulsa, OK, USA	Zirconium dioxide, tricalcium silicate, dimethyl sulfoxide, lithium carbonate, thickening agent.
C-Root SP (C-R)	Beijing C-Root Dental Medical Devices Co. Ltd, China	Zirconium Oxide, strontium silicates, calcium Phosphates, calcium hydroxide, tantalum Oxide, and filler agents.
iRoot SP (i-R)	Innovative BioCeramix Inc., Vancouver, Canada	Zirconium oxide, calcium silicates, calcium hydroxide, calcium phosphate monobasic and filler agents.

percentage of control using the formula: $(A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$ (where A = absorbance).

Osteogenic differentiation

MC3T3-E1 pre-osteoblasts were plated at a density of 1×10^4 cells per well in 12-well plates and cultured in α-MEM for 2 days. α-MEM was then replaced with sealer extracts diluted in the osteogenic medium at a ratio of 1:5. The osteogenic medium consisted of α-MEM supplemented with 10 mmol/L β-glycerophosphate, 50 mg/mL ascorbic acid and 100 nmol/L dexamethasone (Sigma-Aldrich).

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured on days 7 and 14 to assess osteogenic potential, with an osteogenic medium change every 3 days. After removing the culture medium, cells were rinsed with cold phosphate-buffered saline (PBS) and lysed with Assay Buffer (Abcam, Cambridge, MA, USA) on ice. Following centrifugation, the supernatants were analysed using an ALP activity assay kit (Abcam). ALP activity was normalized to total cellular protein concentration as determined by the Bicinchoninic Acid Assay (Thermo Fisher Scientific).

Alkaline phosphatase staining

After 7 and 14 days, the culture medium was removed, and the MC3T3-E1 pre-osteoblasts were washed 3 times with cold PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After washing the samples with cold PBS twice, fixed cells were treated with 1 mL ALP staining reagent (1-step NBT/BCIP solution; Thermo Fisher Scientific) for 60 minutes. After removing the staining reagent, images were captured using a digital camera (Canon, Hong Kong, China).

Alizarin Red S staining

After 21 days of osteogenic induction, calcium mineralization was detected by Alizarin Red S (ARS) staining. The culture medium was removed, and the MC3T3-E1 pre-osteoblasts were washed three times with cold PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Specimens were washed with cold PBS twice and stained with 1% ARS (Abcam) at room temperature. After additional rinsing with PBS, the plates were air-dried, and images of stained nodules were captured using an optical microscope (LV100POL, Nikon, Japan) and a digital camera (Canon). The positively stained areas were quantified using ImageJ software (V1.8.0.112; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction

After 14 days of osteogenic differentiation, the total RNA was extracted from each group of cells using the RNeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime, Shanghai, China). The extracted total RNA was reverse-transcribed into cDNA using PrimeScript™RT Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Specific primer sequences used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) are listed in Table 2. β-Actin served as a housekeeping gene for normalization of gene expression. RT-qPCR was performed with TB Green Premix Ex Taq (Takara Bio) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Grand Island, NY). Cells cultured without sealer material extracts were used as controls. The relative expression of mRNA was normalized to the expression of β-Actin.

Table 2 – List of primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequences (5'-3')
ALP	F: TACATTCCCCATGTGATGGC
	R: ACCTCTCCCTTGAGTGTGGG
β-Actin	F: TGGATGGCTACGTACATGGCTGGG
	R: TTCTTTGCAGCTCCTTCGTTGCCG
COL1A1	F: CATGTTCAGCTTTGTGGACCT
	R: GCAGCTGACTTCAGGGATGT
RUNX2	F: GACTGTGGTTACCGTCATGGC
	R: ACTTGGTTTTTCATAACAGCGGA

ALP, alkaline phosphatase; β-Actin, beta-actin; COL1A1, collagen type I alpha 1; RUNX2, Runt-related transcription factor 2.

Statistical analysis

All experiments were performed in triplicate and repeated three times. Data are presented as the mean ± standard deviation. The normality of the data was tested using the Shapiro-Wilk test. Data were assessed by one-way/two-way ANOVA followed by the Tukey post-hoc test for multiple comparisons using GraphPad Prism 10.1.1 software (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $P < .05$.

Results

Cell viability

Cell viability was assessed on days 3 and 7 using different sealer dilutions (Figure 1A). On day 3, all sealers exhibited significant toxicity at the 1:1 dilution ($P < .001$). AHP showed the highest cytotoxicity across all dilutions, as demonstrated by relative viability of 4.87 ± 1.63% (1:1), 19.98 ± 4.43% (1:2), 75.69 ± 3.92% (1:5) and 84.55 ± 8.50% (1:10) ($P < .001$). At the 1:5 and 1:10 dilutions, C-R, i-R, and AHPbcs showed no cytotoxicity. At the 1:10 dilution, C-R and i-R displayed significantly superior biocompatibility, as indicated by cell viability of 121.52 ± 5.52% (C-R) and 132.78 ± 0.86% (i-R) relative to the control ($P < .001$). On day 7, all sealers remained cytotoxic at the 1:1 dilution ($P < .0001$). AHP was significantly more cytotoxic at the 1:2 dilution (38.42 ± 0.83%, $P < .0001$), while other sealers exhibited similar viability relative to the control ($P > .05$). At the 1:5 dilution, all sealers approached 100% relative cell viability; however, AHP remained significantly cytotoxic compared to the other sealers (95.88 ± 1.45%, $P < 0.001$). At the 1:10 dilution, there were no statistically significant

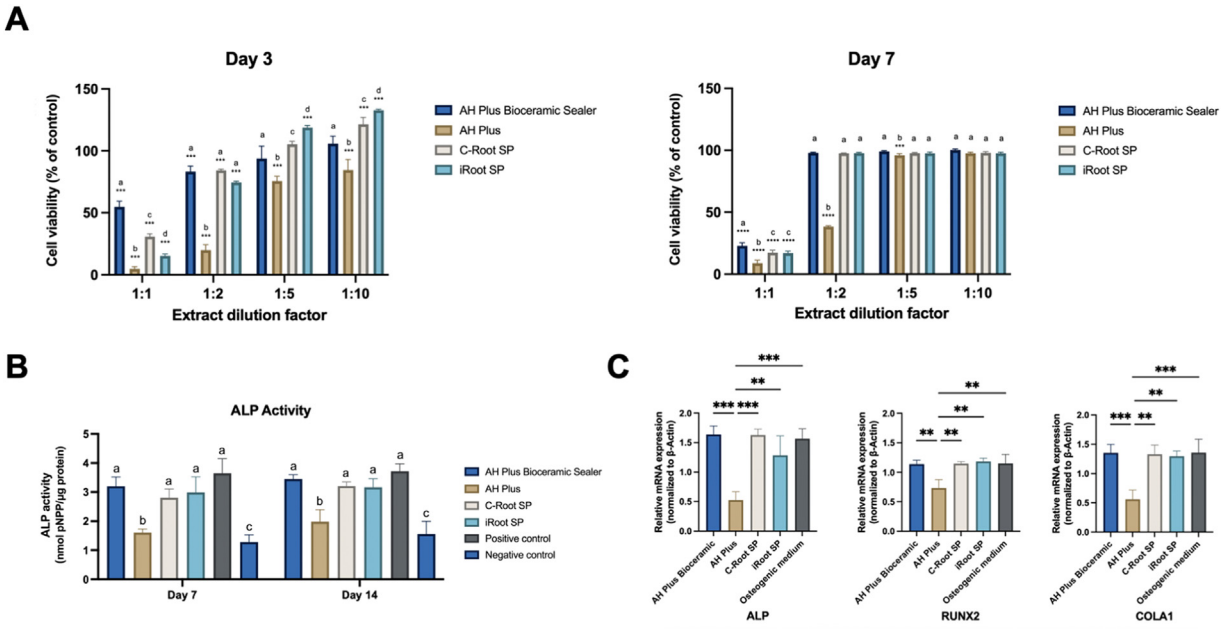

Fig. 1 – A, Cell viability (% of control) of MC3T3-E1 pre-osteoblasts after exposure to serially diluted extracts of AHPbcs, AHP, C-R, and i-R for 3 and 7 days determined by CCK-8 assay. Different letters represent significant differences between groups in each dilution extract. Asterisks indicate significant differences from the control group. Two-way analysis of variance followed by a Tukey post-hoc test (*P < .001; ****P < .0001). B, ALP enzymatic activity assay expressed as nmol pNPP/μg of total cellular protein after 7 and 14 days of exposure to sealer extracts of AHPbcs, AHP, C-R, and i-R at 1:5 dilution. Different letters represent statistically significant differences. One-way analysis of variance followed by a Tukey post-hoc test (P < .0001). C, Relative mRNA expression for ALP, RUNX2, and COL1A1 in MC3T3-E1 pre-osteoblasts after 14 days of exposure to sealer extracts of AHPbcs, AHP, C-R, and i-R at 1:5 dilution, and osteogenic medium (control). Asterisks indicate significant differences from the control group. One-way analysis of variance followed by a Tukey post-hoc test (**P < .01; ***P < .001).**

differences in cell viability between the sealers and the control ($P > 0.05$).

ALP activity

ALP activity was evaluated on days 7 and 14 (Figure 1B). C-R, i-R, and AHPbcs exhibited similar ALP activity levels to the osteogenic medium control ($P > .05$), while AHP showed significantly lower ALP activity than C-R, i-R, and AHPbcs at both time points ($P < .01$). All sealers demonstrated a slight increase in ALP activity from day 7 to day 14.

Reverse transcription-quantitative polymerase chain reaction

After 14 days of exposure to 1:5 sealer extract concentration, the relative mRNA expression of ALP, COL1A1, and RUNX2 was measured (Figure 1C). C-R, i-R, and AHPbcs showed comparable osteogenic expression to the control for all target genes. AHP-treated cells demonstrated significantly lower marker expression than the control group ($P < .01$).

ALP staining

On day 7, C-R, i-R, and AHPbcs showed comparable ALP staining intensity to the osteogenic medium control, while AHP exhibited significantly weaker staining (Figure 2A). By day 14, all groups showed increased ALP staining intensity, with C-R, i-R, and AHPbcs maintaining comparable intensity and distribution. AHP continued to show lower intensity than the other sealers (Figure 2A).

Alizarin Red S staining

Among all sealer extracts, the cells cultured with the sealer extracts of C-R, i-R, and AHPbcs displayed notably more intensely stained mineralized nodules, which were slightly more remarkable compared to the positive control cells cultured in the osteogenic medium (Figure 2B and C). In contrast, the AHP group exhibited fewer mineralized nodules (Figure 2B and C). Quantification of the positively stained nodules indicated that the C-R, i-R, and AHPbcs extracts significantly enhanced mineralization compared to the AHP group ($P < .0001$) (Figure 2D).

Discussion

The development of calcium silicate-based sealers represents a promising advancement in dental materials that may help improve root canal treatment's long-term prognosis. MC3T3-E1 pre-osteoblasts provide a valuable in vitro model to study the influence of sealers on periapical healing.[15,17-19] CCK-8 cytotoxicity assay revealed that all sealers initially showed high toxicity at concentrated dilutions, while C-R, i-R, and AHPbcs became non-cytotoxic at five- and ten-fold dilutions. The cytotoxicity of AHP has been demonstrated in previous

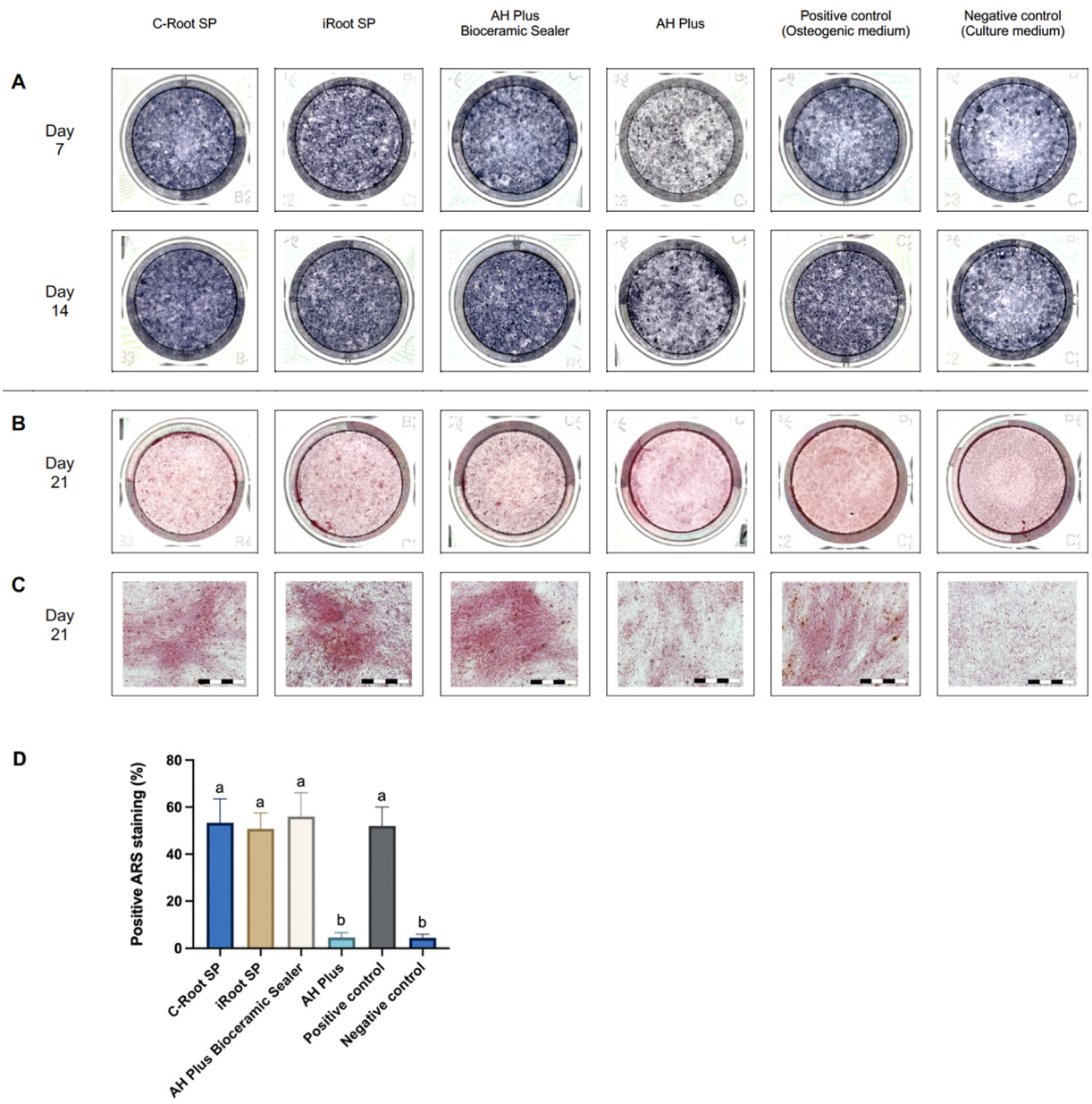

Fig. 2 – A, ALP staining as an early marker of osteogenic differentiation in MC3T3-E1 pre-osteoblasts after 7 and 14 days of exposure to sealer extracts AHPbcs, AHP, C-R, and i-R at 1:5 dilution, osteogenic medium (positive control), and culture medium (negative control) in a 12-well plate. B, ARS staining demonstrating calcium deposits formation after 21-day exposure of MC3T3-E1 pre-osteoblasts to sealer extracts of AHPbcs, AHP, C-R, and i-R at 1:5 dilution, osteogenic medium (positive control), and culture medium (negative control) in a 12-well plate. C, Light microscopy images of ARS-stained mineralized nodules (original magnification: 20 ×; scale bar = 100 μm) after 21-day exposure of MC3T3-E1 pre-osteoblasts to sealer extracts of AHPbcs, AHP, C-R, and i-R at 1:5 dilution, osteogenic medium (positive control), and culture medium (negative control). D, Densitometric quantification of ARS-stained nodules after 21-day exposure of MC3T3-E1 pre-osteoblasts to sealer extracts of AHPbcs, AHP, C-R, and i-R at 1:5 dilution, osteogenic medium (positive control), and culture medium (negative control). Different letters represent statistically significant differences. One-way analysis of variance followed by a Tukey post-hoc test ($P < .0001$).

studies, possibly related to the release of epoxy resin and amine components during the initial setting[20,21] and the production of inflammatory mediators, e.g., cyclooxygenase-2 and nitric oxide synthase, contributing to inflammation in the surrounding tissues.[22] The initial lower degree of biocompatibility of AHPbcs could be attributed to a lower calcium silicate content than i-R.[23] Both strontium silicate and calcium silicate-based sealers showed a comparable cell survival rate, and their cytotoxicity was concentration-dependent. Nevertheless, they were biocompatible at higher dilutions, and the sealer extract at 1:5 dilution was selected in subsequent experiments examining the osteogenic potential to

eradicate the risk of cell apoptosis due to overly high concentrations.

ALP and ARS staining were used to assess and compare osteogenic activity visually. In contrast, the ALP assay and RT-qPCR were adopted to quantitatively measure osteogenic markers, indicating the level of osteogenic differentiation activity. Results showed that cells cultured with C-R, i-R, and AHPbcs expressed significantly higher ALP activity than AHP and culture medium. In addition, the increased ALP staining and the higher concentration of mineralized nodules from ARS staining provided evidence that both calcium silicate-based and strontium silicate-based sealers showed better osteogenic potential than AHP in the cell culture model. RT-qPCR showed that strontium silicate and calcium silicate-based sealers upregulated the expression of key osteogenic markers, ALP, RUNX2, and COL1A1, after 14 days of co-culture. These consistent results indicate that strontium silicate and calcium silicate-based sealers positively promoted osteogenic differentiation and matrix mineralization.

The results revealed calcium silicate-based and strontium silicate-based sealers exhibit good biocompatibility and osteogenic activity compared to the resin-based sealer, and these findings align with previous studies.[19,24,25] AHP was compared due to its long history of clinical use and extensive evidence supporting its use as a control in previous studies.[19,26,27] The cytotoxicity of AH Plus has been demonstrated in previous studies,[19,28,27] which can be attributed to the release of amine and epoxy resin components.[20] i-R and AHPbcs are calcium silicate-based sealers that have shown superior biocompatibility.[27] The biocompatible interactions between calcium silicate-based sealers and host tissues are mainly due to the hydration process of calcium silicates, which releases biologically active ions like calcium and hydroxyl ions, and the formation of calcium phosphate promotes bioactivity and tissue repair.[10,29,30] AHPbcs, as a relatively recently developed material, has not yet been studied with osteoblasts, making it a suitable candidate for our study, in which its reliability can be compared to other calcium silicate-based materials. Our results indicated that AHPbcs exhibited superior biocompatibility with pre-osteoblasts, which is consistent with that of human periodontal ligament stem cells, as shown in a recent study.[25] On the other hand, the biocompatibility and osteogenic potential of C-R demonstrated in this study could be attributed to the content of strontium compounds. Strontium ions are suggested to stimulate osteogenesis and inhibit osteoclastic activity and have prompted research interest in their potential therapeutic applications in regenerative medicine.[31,32] Previous studies have demonstrated enhanced bioactivity through increased ALP activity and hydroxyapatite precipitation when substituting calcium with strontium in bioactive glass.[31,33] Interestingly, strontium ions showed the potential to enhance the differentiation and osteogenic capabilities of human dental pulp stem cells, suggesting its clinical use in promoting pulpal repair.[34] Our results indicated that C-R enhances osteoblast proliferation and the expression of osseous markers, suggesting that incorporating strontium ions into root canal filling materials may improve periradicular tissue repair and regeneration. This finding also concurs with an earlier investigation that explored the effects of C-R using human periodontal ligament stem cells in a root canal-filling model.[24]

While the study yields promising results for the strontium silicate-based sealers, it is important to acknowledge the limitations of this in vitro study. Our study assessed the cytotoxicity of the endodontic sealers after setting for 3 days, which may not reflect clinical scenarios where fresh or unset sealer can be extruded into the periapical tissues inadvertently during obturation. Two-dimensional monolayer cultures also cannot represent cell-to-cell and cell-to-material interactions compared to 3D cell culture models.[35] Furthermore, there is limited evidence and a lack of long-term data to support the clinical use of strontium silicate-based sealers, highlighting the need for clinical studies to validate their impact on endodontic outcomes. Besides, the biocompatibility of endodontic sealers depends not only on the initial cytotoxicity but also on the long-term sustainability of this cytotoxic effect, primarily influenced by the solubility characteristics of the sealers.[36] In addition, it has been suggested that calcium silicate-based sealers exhibit higher solubility and water sorption than epoxy resin-based sealers.[36,37] This calls for future research to investigate the physical properties of strontium silicate-based sealers, as these properties may have profound impacts on their sealing ability and endodontic outcomes. Within the limitations of the study, it can be concluded that C-R, i-R, and AHPbcs are biocompatible with MC3T3-E1 pre-osteoblasts at diluted concentrations and can enhance osteogenic differentiation.

Author contributions

D.C.Y.K.: Methodology, Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing; M.H.: Methodology, Investigation, Visualization, Formal analysis, Writing – review & editing; J.L.: Methodology, Investigation, Visualization, and Formal analysis; C.Z.: Conceptualization, Methodology, Formal analysis, and Writing – review & editing, Supervision; A.H.C.L.: Conceptualization, Methodology, and Writing – review & editing, Supervision; J.W.W.C.: Conceptualization, Methodology, and Writing – review & editing, Supervision.

Conflict of interest

None disclosed.

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