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ORIGINAL ARTICLE

Osteoinductive and regenerative potential of premixed calcium-silicate bioceramic sealers on vascular wall mesenchymal stem cells

Chiara Bernardini[1] | **Fausto Zamparini**[2,3] | **Carlo Prati**[2] | **Roberta Salaroli**[1] | **Andrea Spinelli**[2] | **Augusta Zannoni**[1] | **Monica Forni**[4,5] | **Maria Giovanna Gandolfi**[3]

[1]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy

[2]Endodontic Clinical Section, Dental School, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

[3]Laboratory of Green Biomaterials and Oral Pathology, Dental School, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

[4]Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy

[5]Health Sciences and Technologies-Interdepartmental Center for Industrial Research (CIRI-SDV), Alma Mater Studiorum—University of Bologna, Bologna, Italy

Correspondence
Fausto Zamparini, Endodontic Clinical Section, Dental School, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna 40125, Italy.
Email: fausto.zamparini2@unibo.it

Funding information
Università di Bologna

Abstract

Aim: The osteogenic potential of new premixed calcium-silicate-containing bioceramic sealers (Ca-Si sealers) was tested with porcine vascular wall-mesenchymal stem cells (pVW-MSCs).

Methodology: Two Ca-Si-containing sealers: Ceraseal (MetaBiomed, Cheong-si, South Korea) and AH Plus Bioceramic (Maruchi, Wonju-si, South Korea), and an epoxy resin sealer (AH Plus; Dentsply, Konstanz, Germany) as a control, were prepared according to the manufacturers' indications. All samples were allowed to set for 100% of their setting time in a sterile humid cabinet at 37°C and 95% relative humidity. pVW-MSC seeding efficiency and osteogenic differentiation were analysed as marker of gene/protein expression for up to 12 days. Mineralization assay and immunofluorescence staining were performed and evaluated over a period of 21 days. Statistical analyses were conducted using one-way analysis of variance ($p < .05$). Additional samples were prepared and stored under the same conditions and inspected using an environmental scanning electron microscope equipped with an energy dispersive X-ray spectroscopy system.

Results: Significantly higher cell seeding efficiency ($p < .05$) was observed for both Ca-Si sealers from day 8. pVW-MSCs showed a significant shift towards the osteogenic lineage only when seeded in contact with Ca-Si sealers. Gene expression of osteopontin was upregulated significantly. Collagen I and osteocalcin were clearly expressed by cells in contact with Ca-Si sealers. Mineralization granules were observed in Alizarin red assays and confocal laser scanning microscopy analysis of both Ca-Si sealers. No gene expression or granule mineralization were observed on the epoxy resin sealer.

Conclusions: Premixed Ca-Si sealers displayed a higher potential for osteogenic activity on pVW-MSCs. Epoxy resin sealer was unable to induce any osteogenic activity. The properties of both Ca-Si sealers suggest their potential as osteoinductive platforms for vascular MSCs in periapical bone.

KEYWORDS

Bioactive CaSi, Calcium silicate sealer, CLSM, endodontic sealer, ESEM-EDX, Osteoinductive properties, Vascular-Wall mesenchymal stem cells, VW-MSCs

INTRODUCTION

The presence and persistence of an inflammatory status at the periapical area is triggered by a bacterial infection in the root canal that induces periapical lesion formation (García et al., 2007; Karamifar et al., 2020; Nair, 2006; Ricucci & Siqueira, 2010; Ricucci et al., 2018). Periapical lesions have a high occurrence worldwide and may result in larger bone defects if left untreated (Tibúrcio-Machado et al., 2021).

Previous histological and immunohistochemical studies have indicated the presence of a large number of multipotent cells and mesenchymal stem cells (MSCs) in the periapical area (Liao et al., 2011), such as human periapical cyst MSCs (Marrelli et al., 2013) and human stem cells from periapical papilla (Xue et al., 2023). This area is also characterized by a large vascularization network, similar to those of periodontal ligaments (Davey et al., 2008). Vascular wall-MSCs (VW-MSCs) have therefore been detected in the periapical area (Estrela et al., 2019; Leonardi et al., 2003; Virtej et al., 2013).

VW-MSCs have been described as the main perivascular multipotent populations in blood macro- and micro-vessels (Abedin, Tintut & Demer, 2004; Howson et al., 2005; Invernici et al., 2007; Iurlaro et al., 2003). These cells remain viable in inflamed periapical structures, such as large periapical lesions, and coincide with the vascularization and establishment of both periapical granuloma and periapical cysts (Estrela et al., 2019). The roles of VW-MSCs in new bone formation and bone regeneration processes have been reported in several studies. VW-MSCs participate directly in bone formation and repair (Negri et al., 2020; Pasanisi et al., 2019), as well as indirectly in bone repair via interaction with native skeletal cells (Hong et al., 2022).

The differentiation of MSC populations may require or may be enhanced by the presence of biomaterials capable of supporting their osteogenic activity (Chen et al., 2016; Feng et al., 2023). Previous investigations have reported the beneficial activities of several biomaterials on MSC differentiation towards osteoblasts, in particular, calcium-containing compounds, such as calcium phosphates (Viti et al., 2016; Wang et al., 2014), bioactive glasses (Turner et al., 2023) and calcium silicates (Rathinam et al., 2021; Sun et al., 2009).

Premixed calcium-silicate (Ca-Si)-containing sealers were introduced in the late 2000s and are often referred to as *bioceramic* sealers (Aminoshariae et al., 2022; Camilleri et al., 2022; Donnermeyer et al., 2019 and 2022; Primus et al., 2019). The first premixed bioceramic sealers derived from hydraulic Ca-Si-based cements were composed of tricalcium silicate (up to 35%), dicalcium silicate (up to 15%), thickening agents, and radiopacifiers (up to 45%) and no aluminates and were clinically proposed for cold-filling obturation techniques (Cardinali & Camilleri, 2023; Donnermeyer et al., 2019; Primus et al., 2019).

Extensive data are available on the biological and biomechanical properties of Ca-Si-based cements. These materials release calcium and nucleate apatite deposits when immersed in simulated body fluids (bioactivity) and have the capacity to remineralize dentin collagen (Gandolfi, Parrilli, et al., 2013; Lozano-Guillén et al., 2022; Tay et al., 2007). Additionally, they offer optimal biocompatibility with different cells (Elsayed et al., 2021; Gandolfi et al., 2010; Gandolfi, Shah, et al., 2011; Gandolfi, Taddei, et al., 2011; Niu et al., 2014; Taddei et al., 2009; Tay et al., 2007). Studies on experimental flowable Ca-Si sealers have demonstrated good sealing ability after insertion into the root canal using the carrier-based gutta percha system (Gandolfi, Taddei, et al., 2013). Further investigations have supported their use for wide root apexes (Aminoshariae et al., 2022; Camilleri et al., 2022; Donnermeyer et al., 2019; Primus et al., 2019).

All premixed flowable Ca-Si sealers are characterized by different chemical compositions of radiopacifiers and thickening agents and possess varying percentages of tricalcium silicate (the bioactive component) (Camilleri et al., 2022; Donnermeyer et al., 2022; Primus et al., 2019; Zamparini et al., 2022). Ceraseal, (MetaBiomed, Cheong-si, South Korea) is a recently marketed Ca-Si sealer that is comprised of tricalcium silicate (20%–30%), dicalcium silicate (1%–10%), tricalcium aluminate (1%–10%), zirconium dioxide (45%–50%), and trace amounts of thickening agents. AH Plus Bioceramic (Maruchi, Wonju-si, South Korea), is another recently marketed premixed Ca-Si-containing sealer. Its composition consists mainly of zirconium dioxide (50%–70%) for radiopacity, tricalcium silicate (10%–15%) as a bioactive component, dimethyl sulfoxide (5%–15%), lithium carbonate, and trace amounts of thickening agents.

The chemical–physical properties of these two recently introduced sealers have been investigated in numerous studies (Souza et al., 2023; Zamparini et al., 2022). Alkalizing activity and Ca^{++} release have been demonstrated for both sealers (Souza et al., 2023; Zamparini et al., 2022). The *in vitro* apatite nucleation when

immersed in simulated body solution is modest for these sealers, whereas calcium carbonate formation is predominant (Zamparini et al., 2022).

The cytocompatibility and mineralization potential of these two premixed sealers have been confirmed only for sealer extracts (López-García et al., 2020; Sanz et al., 2022; Shokrzadeh et al., 2023). Additionally, the sealer extracts have been applied towards human periodontal ligament stem cells (López-García et al., 2020; Sanz et al., 2022) and human gingival fibroblast cells (Shokrzadeh et al., 2023).

The contribution to new bone formation processes in the presence of periapical lesions, such as apical granuloma and periapical cysts, is possible due to the availability of bone marrow-derived stem cells and periodontal ligament stem cells, as well as to the ubiquitous presence of vascular MSCs (Estrela et al., 2019) and chronic periapical cyst MSCs (Marrelli et al., 2013). In this context, biomaterials would serve as stable substrates for cell attachment, proliferation, and differentiation into mature osteoblasts. It is therefore important to analyse the behaviour of MSCs when they come into direct contact with fully set sealers.

The porcine model is considered the most suitable animal prototype in the development of stem cell-based therapy, regenerative medicine and transplantation (Ciavarella et al., 2022; Zaniboni et al., 2015), having close similarities to the human physiology and as being more ethically acceptable (Bharti et al., 2016; Zaniboni et al., 2014). Porcine VW-MSCs (pVW-MSCs) (Zaniboni et al., 2014) are able to differentiate not only into the three mesodermal lineages as MSCs isolated from different sources but also as endothelial cells and smooth muscle cells. This extraordinary property makes them a very powerful tool of investigation in many fields including endodontic ones.

The primary objective of the present study was to assess the osteogenic potential of Ceraseal and AH Plus Bioceramic root canal sealers by analysing the resulting osteogenic differentiation protein and gene expression, mineralization activity, and immunofluorescence staining. Additionally, the study delved into the surface morphology and elemental compositions of the constructs. The null hypothesis is the expectation of significant differences in cell attachment, osteogenic differentiation, protein and gene expression, and mineralization of the two premixed sealers, as compared with a standard epoxy resin-based sealer.

MATERIALS AND METHODS

This study has been written according to Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021).

Antibiotic–antimycotic, Phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline (DPBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Trypsin–EDTA solution1X, Dimethyl sulphoxide (DMSO), FluoroshieldTM with DAPI mounting medium and Alizarin red S staining solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pericyte growth medium (PGM) was purchased from Promocell (Heidelberg, Germany). NucleoSpin RNA kit was purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). iTaq Universal SYBR Green Supermix (Cat.172-5121, Bio-Rad laboratories Inc.). All plastic supports for cell culture were purchased from Corning-Beckton-Dickinson (Franklin Lakes, NJ, USA). The sealers composition, formulation, and final setting time are reported in Table 1. The antibodies used in the different applications are reported in Table 2.

ESEM-EDX analyses of set sealers

Sealers were compacted into a mould (1.0 mm diameter, 2.0 mm height, $n = 3$ per composition) to reach 100% of their final setting time at 37°C and 95%RH.

The surface of set sealers was examined by environmental scanning electron microscopy (ESEM; Zeiss EVO 50) with elemental dispersive X-ray microanalysis (EDX; Oxford Instruments). Operative parameters were reported elsewhere (Gandolfi et al., 2010; Gandolfi, Parrilli, et al., 2013; Gandolfi, Taddei, et al., 2011). Microchemical spectra and semiquantitative compositional data were provided through EDX.

Cell culture and treatments

All procedures on pigs were reviewed and approved in advance by the Ethical Committee of the University of Bologna and were then approved by the Italian Ministry of Health (Protocol number n.43-IX/9 all.37; 20/11/2012). Primary pVW-MSCs previously isolated and expanded (Bernardini et al., 2019; Forni et al., 2020) were thawed, seeded and cultured in primary culture flasks (1.5×10^6 cells/T75-flask) in PGM medium with 1× antibiotic–antimycotic solution in a 5%CO_2 atmosphere at 38.5°C. (Bernardini et al., 2019; Forni et al., 2020).

At ~70% confluence, cells were detached with trypsin–EDTA 1X solution, counted and the expression of typical MSC markers was checked before the starting of experiments. To confirm mesenchymal immunophenotyping after freezing, cells thawed and grown in standard culture conditions till 70% of confluence were analysed by flow cytometry as previously described (Bernardini et al., 2019).

All procedures were made in sterile conditions under a laminar flow hood. The sealers were inserted into 1 mL

TABLE 1 Sealer formulation, lot number, composition, and setting time used for the subsequent analyses.

Sealer	Formulation	Lot	Composition	Final setting time
Ceraseal (MetaBiomed Cheong-si, South Korea)	Premixed	CSL2108201	Zirconium dioxide (45%–50%), tricalcium silicate (20%–30%), dicalcium silicate (1%–10%), tricalcium aluminate (1%–10%), thickening agents, polyethylene glycol	660 min
AH Plus Bioceramic (Maruchi Wonju-si, South Korea)	Premixed	KI211103	Zirconium dioxide (50%–70%), tricalcium silicate (5%–15%), dimethyl sulfoxide (10%–30%), lithium carbonate (0.5%), thickening agents (<6%)	720 min
AH Plus (Dentsply Konstanz, Germany)	Paste to paste	2109000972	Paste A: diepoxide, calcium tungstate, zirconium oxide, aerosil, pigment (iron oxide) Paste B: 1-adamantane amine, N,N′-dibenzyl-5-oxa-nonandiamine-1,9, TCD-diamine, calcium tungstate, zirconium oxide, aerosil, silicone oil	1300 min

TABLE 2 Antibody list used for the immunophenotyping of cells and osteogenic differentiation.

Antibody	Product number	Specie	Supplier	Dilution
Primary				
CD45-APC	K252-1E4	Mouse	AbD Serotec	10 μL/10^6 cells
CD90-APC	Ab139364	Mouse	Abcam	10 μL/10^6 cells
CD105-FITC	Ab53318	Mouse	Abcam	20 μL/10^6 cells
CD56-PE	304 606	Mouse	Biolegend	10 μL/10^6 cells
CD44-PerPC	103 036	Rat	Biolegend	10 μL/10^6 cells
CD34-unconjugated	Ab81289	Rabbit	Abcam	1:60
Collagen I	MA1-26771	Mouse	Thermo	1:100
Osteocalcin	MA1-20786	Mouse	Thermo	1:100
Secondary				
Anti-rabbit-PE	Ab97070	Goat	Abcam	1:200
Anti-mouse-FITC	F4143	Goat	Sigma	1:800
Anti-mouse-Alexa fluor 594	A-11032	Goat	Thermo	1 μg/mL

syringes and 0.10 mL/well were dispensed onto a 24 multi-well plate using a 28G needle ($n = 8$ for each formulation). The quantity of the sealer used was calculated to correspond to the sealer disks used in previous laboratory studies (Gandolfi et al., 2010; Gandolfi, Parrilli, et al., 2013; Gandolfi, Taddei, et al., 2011; Zamparini et al., 2022). The diameter of the mould in the previous study was 10 mm, the height was 2.0 mm, and was soaked in 10 mL solution (Zamparini et al., 2022).

The calculated volume ($V = \pi r^2$. h, where V is the volume, r is the radius and h is the height) was 0.157 cm^3 that is 0.157 mL to simulate the same conditions in 10 mL medium. We used this ratio between sealer volume and culture medium in all tests. This ratio agreed with ISO tests that required at least 126.6 mm^2/mL of surface contact (ISO 10993-12:2). The sealer was disposed of at the centre of the well and care was performed to obtain a smooth and regular surface.

Additional samples were prepared by dispensing 0.10 mL/chamber sealer for each formulation onto 8-slide chambers. The unset sealers were positioned along all the available surfaces and maintained in a sterile humidified cabinet at 37°C, 95%RH for 100% of their final setting time (Table 1) (Zamparini et al., 2022).

All the experiments were performed with cells at the sixth passage. Upon their setting, cells were seeded in 24 multiwells or 8-slide chambers previously coated with sealers (1×10^5 /well or chamber).

Cells were maintained in culture for 12 days, the morphology was carefully checked at every medium change by an inverted microscope (Eclipse TS100, Nikon, Tokyo, Japan) equipped with a digital camera (Digital C-Mount Camera TP3100, Kowa, Aichi, Japan). Cell seeding efficiency (CSE) was estimated as previously described (Zaniboni et al., 2015).

RNA extraction and qPCR

RNA extraction was performed using TRI Reagent and the NucleoSpin RNA II kit (Forni et al., 2020) and 500 ng of total high-quality RNA (A260/A280 ratio above 2.0) was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., CA, USA). Swine primers for secreted phosphoprotein 1 (*SSP1*), beta-Actin (*B-ACT*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Sus scrofa ribosomal protein L35 (RPRL 35) were used (Zaniboni et al., 2015). Quantitative real-time PCR was performed to evaluate gene expression profiles in iCycler (Bio-RAD) using SYBR green I detection system. The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis. Gene relative expression was normalized according to the geometric mean of the reference gene (RPLR) (Forni et al., 2020). mRNA relative expression was analysed as fold increase using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), the control group was constituted by pVW-MSCs cultured for 12 days without biomaterials.

Immunofluorescence analysis

To study osteoblast differentiation, cells were washed twice in DPBS and fixed in 4% paraformaldehyde for 30 min. After three washes with DPBS, unspecific epitopes were blocked by incubating with a blocking solution of 0.3% Triton and 2% foetal bovine serum for 1 h at room temperature. Then cells were stained by 24 h incubation at 4°C with appropriate primary antibodies in the blocking solution. The following day, cells were washed three times with DPBS and incubated 1 h at RT with appropriate secondary antibodies (Table 2). Negative controls consisted of the omission of the primary antibody in every run experiment. At the end of the procedure, coverslips were mounted on slides with mounting solution containing 0.1 μg/mL DAPI. Images were acquired using a confocal laser scanning microscope (Nikon A1R, Amstelveen, The Netherlands). Emission peaks were 489 nm for DAPI, 561 nm for osteocalcin and 638 nm for collagen 1 respectively. The acquisition time was 120 s and all images were acquired with at least 1024 resolution.

Alizarin red staining

Staining of the extracellular matrix with Alizarin red S dye assay was performed. The culture medium was carefully removed, and cells were washed twice with PBS. Cells were then fixed with cold 4% paraformaldehyde for 20 min at RT. After washing twice with distilled water, cells were stained by adding Alizarin red S staining solution. After an incubation time of 30 min in the dark, the Alizarin red S staining solution was removed, and the cells were washed twice with distilled water. The staining was analysed under an inverted microscope (Eclipse TS100, Nikon, Tokyo, Japan) equipped with a digital camera (Digital C-Mount Camera TP3100, Kowa, Aichi, Japan).

RESULTS

Environmental scanning electron microscopy–energy dispersive X-ray spectroscopy analyses of set sealers

Backscattered images of the tested sealers are displayed in Figure 1a–f. The set surface of Ceraseal was observed using environmental scanning electron microscopy (ESEM) at 3000× magnification. The surface was uniform and showed numerous electron-dense granules (size range: 2–5 μm) (Figure 1a). Zirconium (Zr) (the radiopacifier), calcium (Ca), silicon (Si) (from calcium-silicate) and a trace of aluminium (Al) were detected (Figure 1b).

ESEM images at 3000× magnification of the AH Plus Bioceramic sealer presented a non-uniform surface with several pits and irregularities (Figure 1c). Energy dispersive X-ray spectroscopy (EDX) analyses indicated the presence of Zr Ca, and Si. There were no traces of sulphur (S) from dimethyl sulfoxide (Figure 1d).

ESEM images at 3000× magnification of AH Plus revealed a uniform surface with few irregularities and

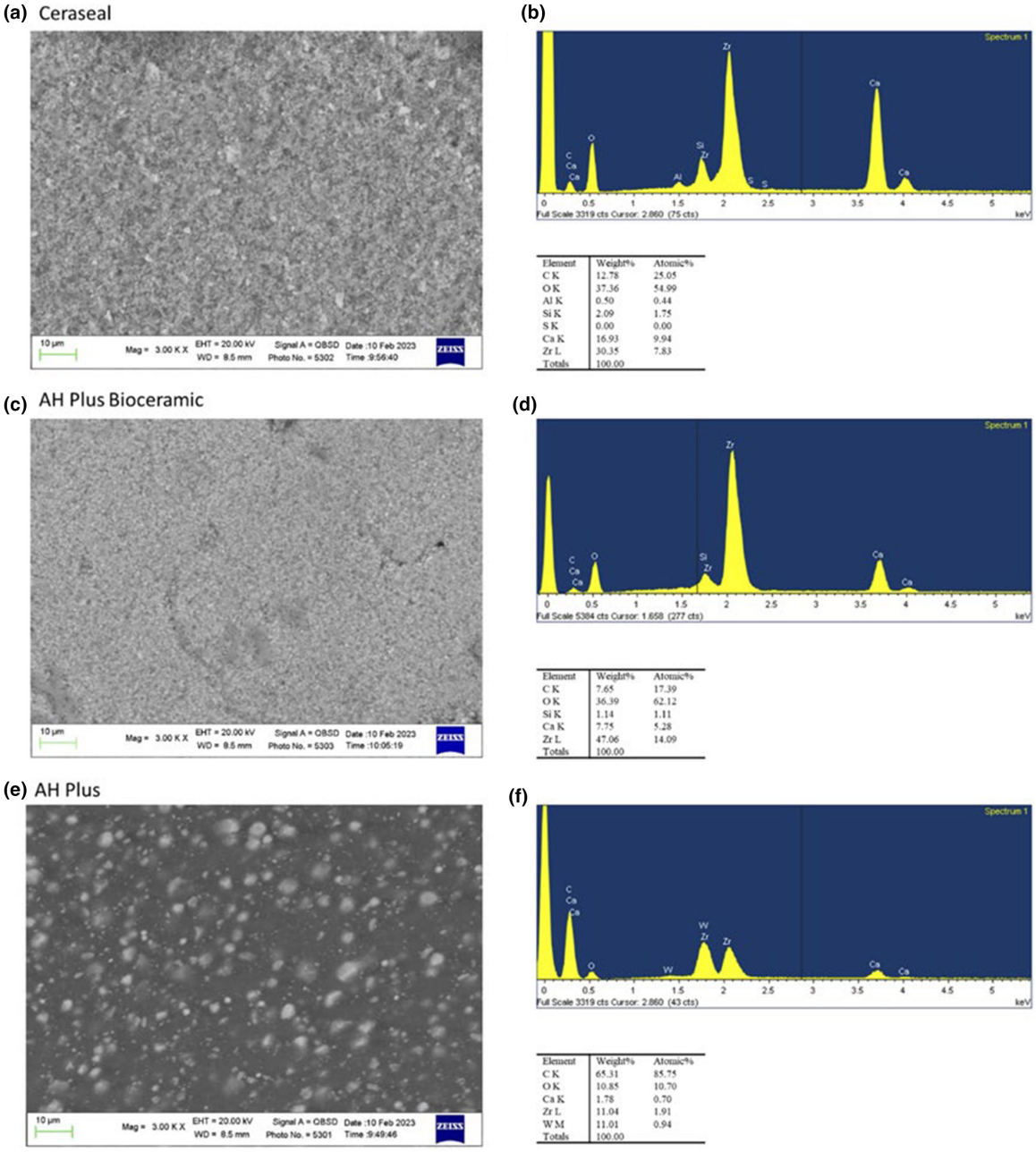

FIGURE 1 (a) ESEM image at 3000× of Ceraseal after 100% of setting time. The surface appeared regular with different electron-dense granules (b) EDX analyses showed the presence of Zr (radiopacifier), Ca and Si (from Ca-Si), Al from calcium aluminates. (c) ESEM image at 3000× of AH Plus Bioceramic after 100% of setting time. The surface was more homogeneous when compared with Ceraseal. (d) EDX analysis showed a higher Zr peak and lower Si and Ca peaks, revealing a lower presence of bioactive Ca-Si in the formulation. (e) ESEM image of AH Plus. The surface appears homogeneous with no superficial granules. (f) EDX analysis revealed the high presence of N and C (from the epoxy resins). The presence of W and Zr was attributable to the radiopacifier used in the formulation. Modest peak of Ca was present.

granules (size range: 5–10 μm) (Figure 1e). The surface showed a greater distribution of low electron-dense areas, related to the high atomic percentage of C (from epoxy resins and amines). The high electron-dense areas were attributable to Zr and wolframium (the radiopacifier) (Figure 1f).

Porcine vascular wall-mesenchymal stem cell (pVW-MSC) characterization

After thawing, 1×10^6 pVW-MSCs seeded in a T75 tissue flask reached confluence in 7 days, with an average doubling time of 39.75 ± 2.21 h, and showed a thin

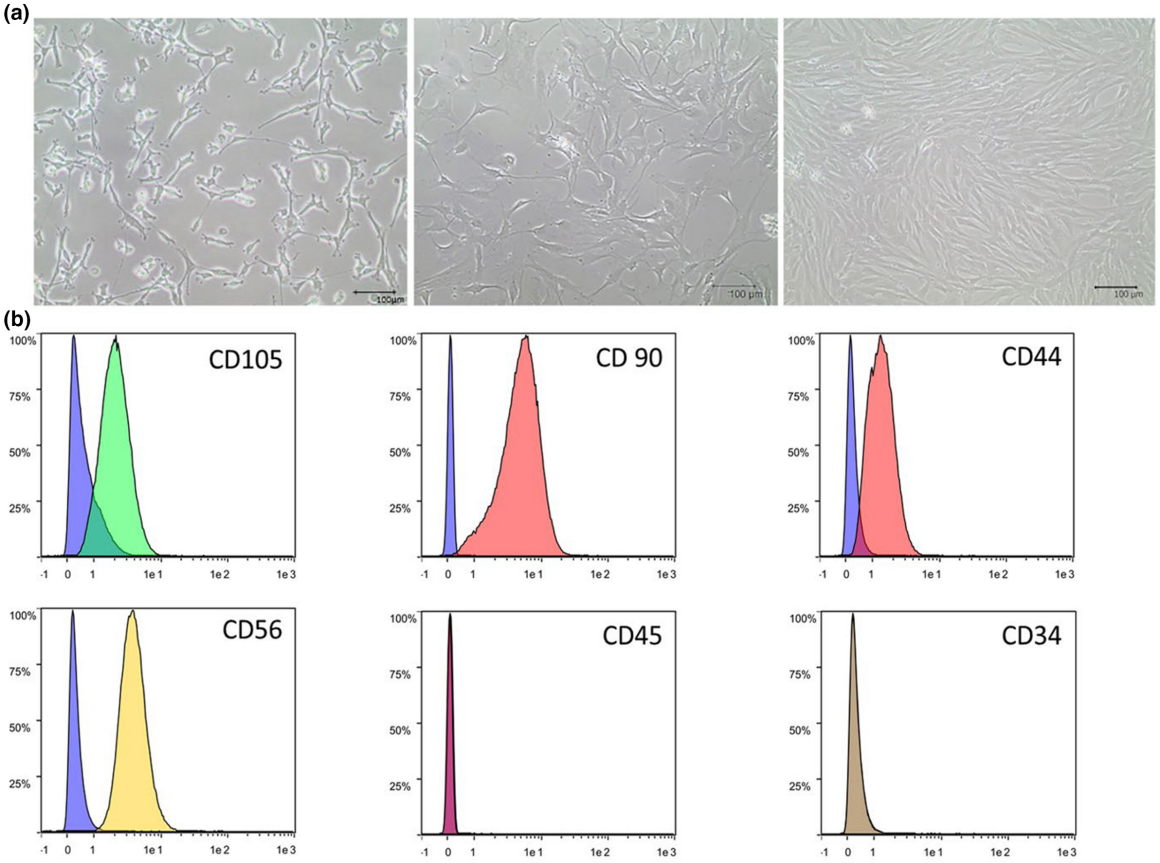

FIGURE 2 Phenotypic analysis of pVW-MSCs after thawing. (a) Representative images of pVW-MSCs morphology at different stage of confluence. Scale bar = 100 μm. (b) Flowcytometric analysis of cell-surface markers in porcine vascular wall mesenchymal stromal cells. Each graph shows the percentage of cells expressing the specific marker reported [cultured area under the curve (AUC)] and the relative negative control (violet AUC, cells not incubated with any antibodies). This analysis confirmed the mesenchymal stromal cell-like immune profile of porcine vascular wall mesenchymal stromal cells: CD105, CD90, CD 44 and CD 56 were highly expressed (>96%) whilst the haematopoietic markers CD45 and CD34 were nearly absent (<2.5%). AUC, Area under the curve.

spindle-shaped morphology, typical of mesenchymal vascular wall stem cells (Figure 2a).

Flowcytometric analysis confirmed an MSC immunophenotype displaying an MSC profile. In line with the criteria for human MSC characterization (Dominici et al., 2006) and with that described for pig MSCs (Bharti et al., 2016), the cell population was positive for markers of mesenchymal stemness: CD105, CD90, CD44, CD56, and negative for haematopoietic markers CD45 and CD34 (Figure 2b).

pVW-MSC growth in the presence of Ca-Si sealers

Cells cultured in the presence of Ceraseal and AH Plus Bioceramic showed adherence at 24 h after seeding (Figure 3a). Cells failed to attach completely in the presence of AH Plus and showed a rounded form as efficient bonds amongst them were not well-established. As of day 8, the cells had become completely detached (Figure 3). Consequently, for cells cultured in the presence of AH Plus, none of the subsequent analyses were carried out. After 12 days of culture in the presence of Ceraseal and AH Plus Bioceramic, the cell morphology had changed from a thin spindle shape to a polygonal form. In addition, the area of overlapping cells appeared at first near the biomaterials, but then spread around with the emergence of a typical 'spherical core'.

Osteogenic differentiation

No significative differences were observed in the cytoskeletal marker beta-actin or the metabolism marker of glyceraldehyde 3-phosphate dehydrogenase expression (Figure 4a,b). In contrast, the osteopontin gene expression (SPP1) was significantly increased in cells cultured in both biomaterials, with a greater increase of

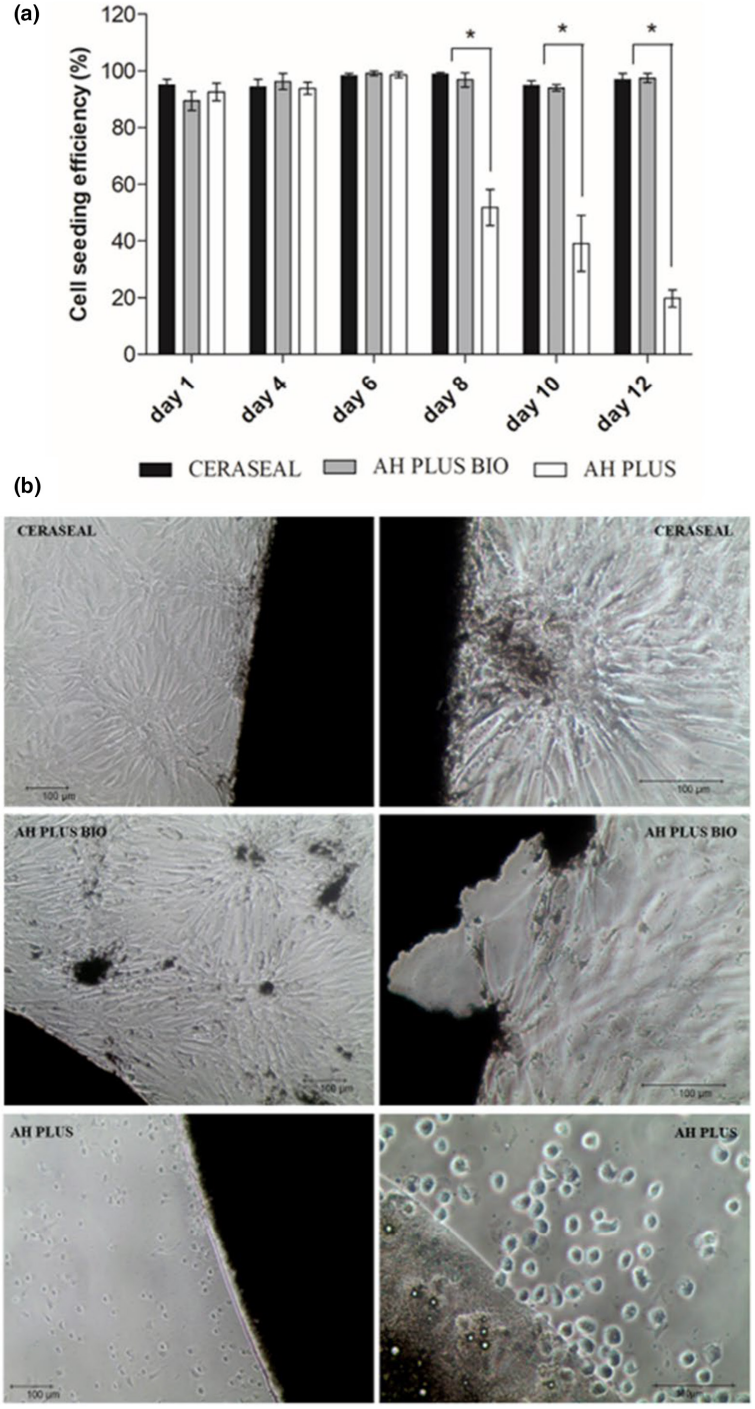

FIGURE 3 pVW-MSCs growth with bioceramic sealer. (a) Cell seeding efficiency. After 24 h from the seeding on the scaffold surface, the unattached cells were quantified and cell seeding efficiency was calculated by the equation: CSE (%) = (1 − cellsu/cellsi) × 100. Data represent the mean ± SD of three independent biological replicates ($n = 3$) and were analysed using one-way ANOVA followed by the Tukey's post hoc comparison. Significant differences amongst the experimental scaffolds are represented by asterisks (*) ($p < .05$). (b) Representative images of pVW-MSC morphology when cells were growing with Ceraseal, AH Plus Bioceramic and AH Plus scale bar: 100 μm.

50-fold in Ceraseal (Figure 4c), compared with the cells cultured in the absence of bioceramic sealers (i.e. the control group, CTR).

Immunofluorescence staining showed clear positivity to collagen 1A (Figure 5a–c) and osteocalcin (OCN) (Figure 5d–f) for cells cultured in the presence of Ceraseal or AH Plus Bioceramic. No signal was detected in cells cultured in the absence of bioceramic sealers. Alizarin red staining (Figure 5g–l) displayed red mineralized deposits both for cells grown in the presence of Ceraseal and AH Plus Bioceramic; notably, spherical cores were evident with both sealers (Figure 5h,i,k,l).

Two representative spherical cores are shown in Figure 6a,b. The analysis revealed that cells formed a well-organized three-dimensional structure surrounding the calcium-rich spherical core. The cells exhibited a high degree of interconnectedness and expressed OCN, indicating their active involvement in the mineralization process.

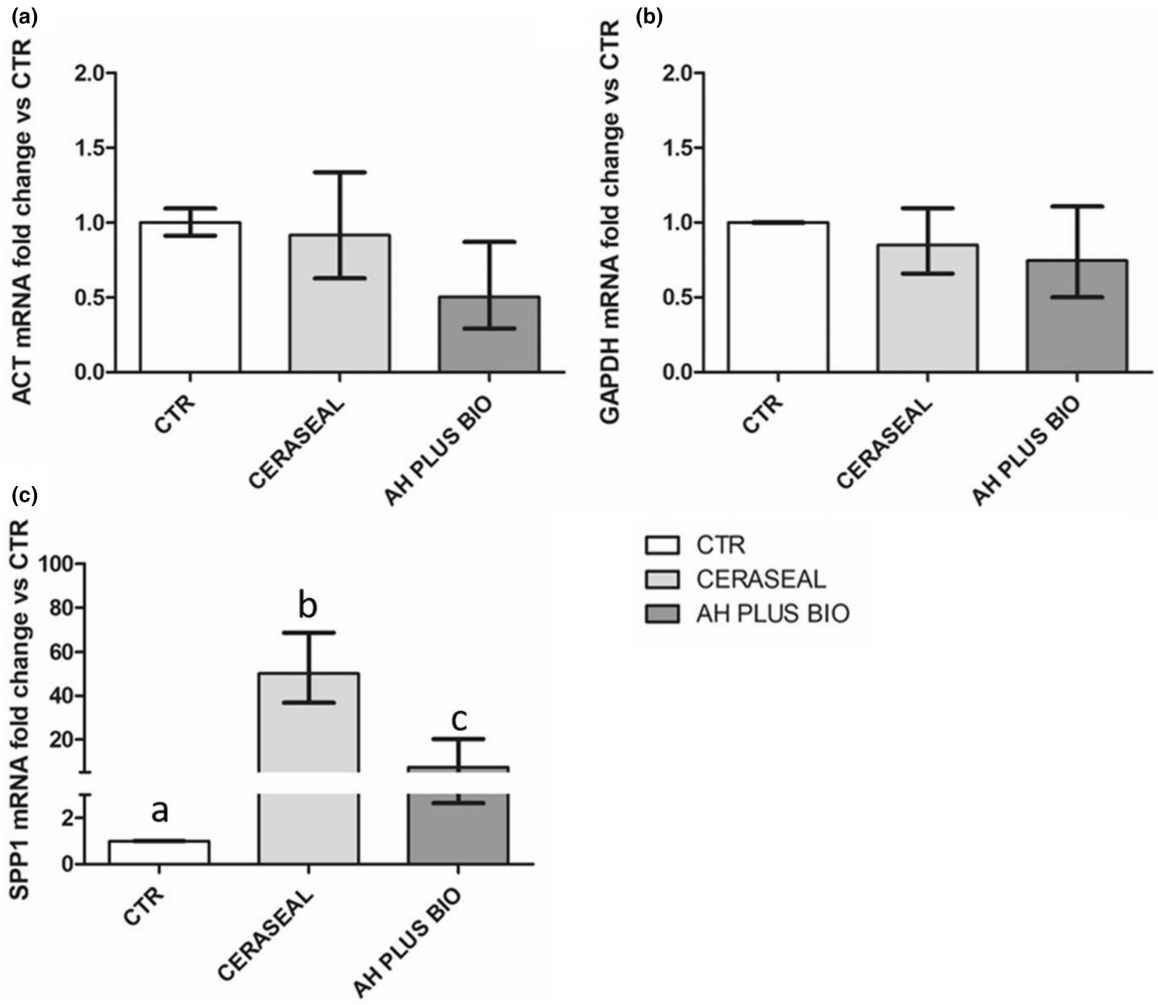

FIGURE 4 Effect of biomaterials on gene expression of housekeeping ACT (a), GAPDH (b) and osteogenic marker SPP1 (c). The SPP1 expression increased in both biomaterials with different levels of increase: 50 and 7 more times in Ceraseal and AH Plus Bioceramic respectively. The data are reported as a fold of change with respect to cell culture in standard conditions. Different letters indicate statistically significant differences ($p < .05$, ANOVA post hoc Tukey test) amongst groups.

DISCUSSION

Our results showed that both premixed Ca-Si sealers (Ceraseal and AH Plus Bioceramic) supported pVW-MSC surface colonization and induced their differentiation into osteoblasts, as shown by cell seeding and osteogenic differentiation assays. It is important to note that pVW-MSCs were cultured directly in contact with set sealers and without adding any osteogenic differentiation supplements to the medium.

Significant mRNA expression of osteopontin by pVW-MSCs was observed when cultured in contact with the two Ca-Si sealers. Osteopontin is a critical osteodifferentiation marker, and it is expressed in both early and late stages of osteoblast differentiation (Huang et al., 2007; Wang & Denhardt, 2008). Immunostaining analysis clearly revealed the positivity of pVW-MSCs to collagen-1 and OCN, both detectable in fully differentiated osteoblasts (Huang et al., 2007; Wang & Denhardt, 2008) cultured on Ca-Si sealers. These differentiated osteoblast cells displayed a polygonal morphology and formed calcium nodules on the Ca-Si sealer surface. The *in vitro* mineralization of the deposits and the spherical cores were revealed by the Alizarin red assay.

The present data are well-supported by the findings from a previous investigation by Sanz et al. (2022). Osteogenic expression and mineralization activity of AH Plus Bioceramic and another Ca-Si sealer extract (Endosequence-BC) on human periodontal ligament stem cells have been demonstrated (Sanz et al., 2022). The same study could not confirm mineralization with AH Plus Bioceramic (Sanz et al., 2022).

Another investigation (Giacomino et al., 2019) showed the osteogenic potential of a premixed Ca-Si sealer (Endosequence-BC) and an MTA Ca-Si based cement (Pro-Root MTA). A comparison of AH Plus and

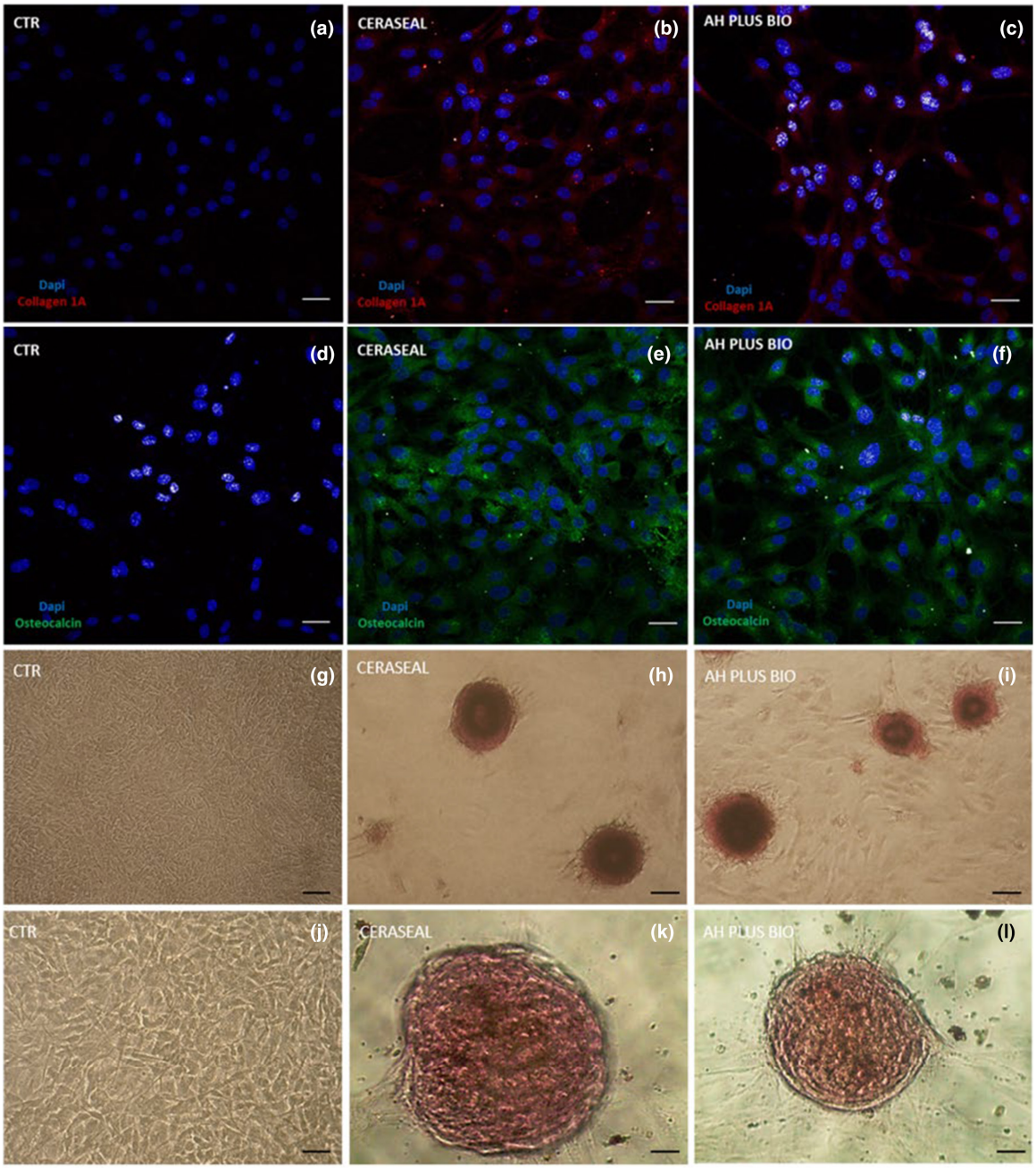

FIGURE 5 Osteogenic differentiation of pVW-MSCs cultured with Ceraseal (b, e, h and k) or AH Plus Bio (c, f, i, l) or in standard culture condition (a, d, g, j). Immunostaining of collagen IA (a–c, red signal) and osteocalcin, (d–f green signal). Nuclei were always counterstained with DAPI (blue signal). Alizarin red staining (g–l) demonstrated the presence of calcium-rich deposits in cells cultured with Ceraseal and AH Plus Bioceramic. CTR, control cells cultured without bioceramic sealers. Scale bar = 10 μm.

zinc-oxide eugenol sealers did not indicate any osteogenic potential (Giacomino et al., 2019). A recent study analysed the osteogenic response of IRoot SP extracts on human stem cells from periapical papilla previously conditioned with osteogenic conditioned medium (Xue et al., 2023); their results revealed the osteogenic differentiation of human stem cells by inhibiting the miR-141-3p pathway.

There was no gene expression analysis of AH Plus, as we observed no cell adhesion on the sealer surface. AH Plus tends to show modest biocompatibility with cells during their setting time (Gandolfi et al., 2008, 2010; Jung et al., 2018; Sanz et al., 2022). AH Plus displayed markedly less/lower cell seeding of <20% from day 8. The pVW-MSCs did not attach in the presence of AH Plus and showed a round morphology without having established

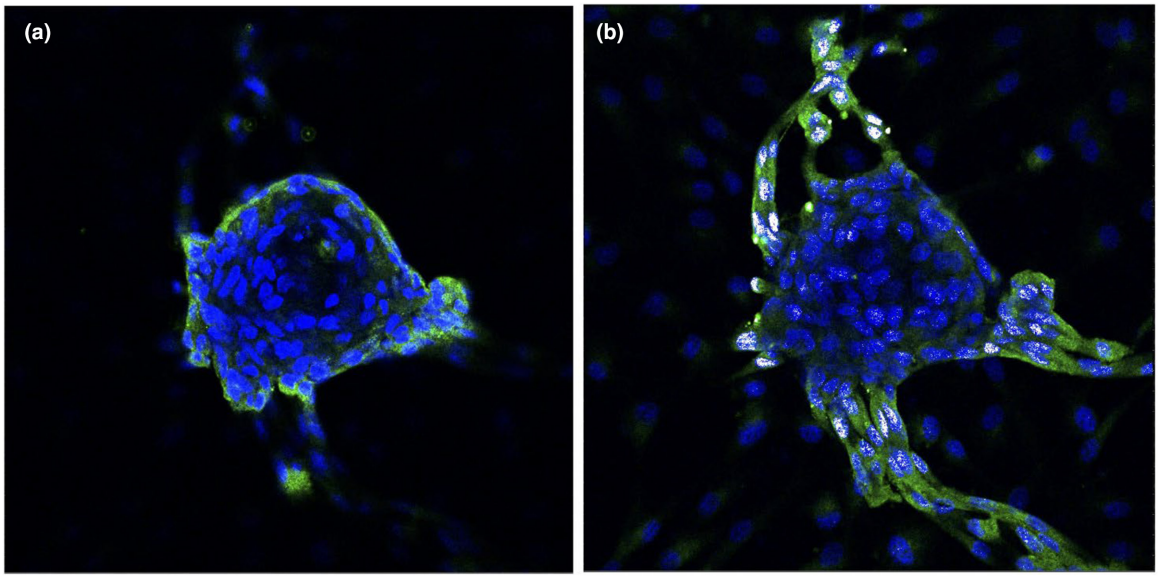

FIGURE 6 CLSM analysis on two (a, b) mineralized spherical cores detected on the AH Plus Bioceramic group. Immunostaining of osteocalcin (green signal) and counterstaining of cell nuclei through DAPI.

efficient bounds. The hydrophobic behaviour of the AH Plus surface may explain the lack of adhesion of the cells on its surface (Lee et al., 2020).

The discrepancies between Ca-Si sealers and non-Ca-Si sealers may be related to the different components and their chemophysical properties. As previously mentioned, both Ca-Si sealers examined in this study released calcium ions during the early stages after setting (Zamparini et al., 2022) and were able to alkalize the medium (Souza et al., 2023; Zamparini et al., 2022). These sealers create the conditions for the formation of a thin surface layer, composed mainly of calcium carbonate and apatite (Zamparini et al., 2022), previously described as bioactivity (Gandolfi et al., 2010; Niu et al., 2014). Bioactivity (apatite nucleation ability) has also been indicated for other Ca-Si sealers, specifically BioRoot-RCS, Total-Fill-BC, and Endosequence-BC, using ESEM–EDX and micro-Raman spectroscopy (Siboni et al., 2017; Zamparini et al., 2019), SEM-EDX (Shokouhinejad et al., 2012), and SEM-EDX with X-ray diffraction (Moinzadeh et al., 2016) analyses, respectively.

Ca-Si bioceramic biomaterials have hydraulic and hydrophilic properties and release silicates (Primus et al., 2019), conditions that may favour MSC adhesion and their proliferation (Sun et al., 2009). Additionally, these materials promote the signalling required for osteodifferentiation (Sun et al., 2009). Calcium ions provide powerful extracellular signals for osteoblast differentiation, as observed in previous investigations of MTA-like materials on several bone-specific cells (Gandolfi et al., 2010; Gandolfi, Shah, et al., 2011; Koutroulis et al., 2019; Rathinam et al., 2021; Santiago et al., 2021). Calcium ions stimulate the expression of bone-associated proteins (Jung et al., 2010) and are necessary for cell differentiation and mineralization. Calcium ions and phosphates from solutions/blood are needed for apatite formation phases (Gandolfi et al., 2010; Gandolfi, Taddei, et al., 2011). It is probable that surface apatite formation supports osteogenic differentiation and induces osteoblasts to produce and mineralize new bone tissue via the deposition of apatite crystals (Gandolfi, Shah, et al., 2011; Schröder et al., 2012; Turner et al., 2023). Silicates have the ability to increase the expression of osteogenesis-related genes such as collagen-type-I, alkaline phosphatase, OCN, Runx2, and the Wnt/-catenin signalling pathway (Santiago et al., 2021; Spencer et al., 2006; Turner et al., 2023; Zou et al., 2009).

Other Ca-Si-based cements, such as MTA-like materials, demonstrate a higher rate of calcium release (Candeiro et al., 2012; Gandolfi, Parrilli, et al., 2013; Gandolfi, Taddei, et al., 2013; Kharouf et al., 2020; Lee et al., 2017) and a stronger ability to increase the pH of solutions (Candeiro et al., 2012; Kharouf et al., 2020; Niu et al., 2014). Short-term clinical data evidenced an acceptable outcome in the first 12–24 months after obturation phases when used with both cold (Gautam et al., 2022; Song et al., 2022) and warm obturation techniques (Pontoriero et al., 2023; Spinelli et al., 2023; Zamparini et al., 2023). Interestingly, these sealers showed a lower extrusion rate (Gautam et al., 2022; Pontoriero et al., 2023; Song et al., 2022; Zamparini et al., 2023) and lower post-obturation pain compared with the gold-standard epoxy resin-based sealers (Song

et al., 2022). The clinical use of the analysed Ca-Si should be supported by well-designed long-term studies to evaluate their long-term dimensional stability when apically extruded or when in the presence of large bone defects (Primus et al., 2022).

This study had some limitations. Freshly mixed materials were not tested, as testing the sealers in their freshly unset state was not feasible due to the intrinsically difficult setting conditions of premixed sealers not following a traditional base catalyst setting reaction. In such cases, sealer dissolution could lead to an unstable surface for cell interaction. In this regard, testing the freshly placed materials would have been useful with respect to testing the initial cytotoxicity of the sealer when placed in contact with the cells, thus simulating the initial reactions (attributable to pH variations and ions released from sealers) that occur in the periapical area immediately upon contact of the sealer with the periapical tissue. The use of extracts from premixed sealers at different concentrations was not considered, as this has previously been performed in a number of studies on both Ceraseal and AH Plus Bioceramic sealers (e.g. Giacomino et al., 2019; Sanz et al., 2022) and because this step is usually performed in preliminary investigations to assess the initial cytotoxicity of the material, or when a high concentration could potentially induce damage to cells (ISO 10993-5:2009).

The use of set sealers was applied to simulate the further biological steps that occur after the sealing application and the biological effect of a potential extrusion in the periapical area. Indeed, our investigations were performed over the course of 21 days, allowing for the observation of strong osteogenic differentiation of VW-MSCs and *in vitro* mineralization.

CONCLUSIONS

The present study demonstrated the capacity of new Ca-Si-containing bioceramic sealers to induce osteodifferentiation of pVW-MSCs. These cells showed osteopontin expression when in contact with the Ca-Si sealers.

AUTHOR CONTRIBUTIONS

Conceptualization, C.P. and C.B.; methodology, C.B, A.Z., R.S., F.Z. and M.G.G.; validation, F.Z. and A.S.; formal analysis, A.Z. and R.S.; investigation, F.Z. and A.S.; resources, C.B.; data curation, A.Z. and R.S.; supervision, C.P., M.G.G and M.F.; writing—original draft preparation, F.Z., M.G.G. and C.B.; writing—review and editing, C.P., M.F. and F.Z.; visualization, F.Z. and A.S.; project administration, M.F. and M.G.G. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures on pigs were reviewed and approved in advance by the Ethical Committee of the University of Bologna and were then approved by the Italian Ministry of Health (Protocol number n.43-IX/9 all.37; 20/11/2012).

ORCID

Chiara Bernardini https://orcid.org/0000-0003-3384-0244
Fausto Zamparini https://orcid.org/0000-0002-0071-4463
Carlo Prati https://orcid.org/0000-0001-8163-8628
Roberta Salaroli https://orcid.org/0000-0002-8819-2857
Andrea Spinelli https://orcid.org/0000-0002-4674-1766
Augusta Zannoni https://orcid.org/0000-0003-0361-7594
Monica Forni https://orcid.org/0000-0003-1310-6202
Maria Giovanna Gandolfi https://orcid.org/0000-0001-7793-6227

REFERENCES

Abedin, M., Tintut, Y. & Demer, L.L. (2004) Mesenchymal stem cells and the artery wall. *Circulation Research*, 95, 671–676.

Aminoshariae, A., Primus, C. & Kulild, J.C. (2022) Tricalcium silicate cement sealers: do the potential benefits of bioactivity justify the drawbacks? *Journal of the American Dental Association*, 153, 750–760.

Bernardini, C., Bertocchi, M., Zannoni, A., Salaroli, R., Tubon, I., Dothel, G. et al. (2019) Constitutive and LPS-stimulated secretome of porcine vascular wall-mesenchymal stem cells exerts effects on in vitro endothelial angiogenesis. *BMC Veterinary Research*, 15, 123.

Bharti, D., Belame Shivakumar, S., Baregundi Subbarao, R. & Rho, G.J. (2016) Research advancements in porcine derived mesenchymal stem cells. *Current Stem Cell Research & Therapy*, 11, 78–93.

Camilleri, J., Atmeh, A., Li, X. & Meschi, N. (2022) Present status and future directions: hydraulic materials for endodontic use. *International Endodontic Journal*, 55, 710–777.

Candeiro, G.T., Correia, F.C., Duarte, M.A., Ribeiro-Siqueira, D.C. & Gavini, G. (2012) Evaluation of radiopacity, pH, release of calcium ions, and flow of a bioceramic root canal sealer. *Journal of Endodontics*, 38, 842–845.

Cardinali, F. & Camilleri, J. (2023) A critical review of the material properties guiding the clinician's choice of root canal sealers. *Clinical Oral Investigations*, 27, 4147–4155.

Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q. et al. (2016) Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death and Differentiation*, 23, 1128–1139.

Ciavarella, C., Valente, S. & Pasquinelli, G. (2022) The characteristics and survival potential under sub-lethal stress of mesenchymal stromal/stem cells isolated from the human vascular wall. *Stem Cells*, 31(40), 1071–1077.

Davey, K.J., Perrier, S., Ohe, G., Gilbert, A.D., Bankfalvi, A., Saunders, W.P. et al. (2008) Assessment of vascularity as an index of angiogenesis in periradicular granulomas: comparison with oral carcinomas and normal tissue counterparts. *International Endodontic Journal*, 41, 987–996.

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, 315–317.

Donnermeyer, D., Bürklein, S., Dammaschke, T. & Schäfer, E. (2019) Endodontic sealers based on calcium silicates: a systematic review. *Odontology*, 107, 421–436.

Donnermeyer, D., Schemkämper, P., Bürklein, S. & Schäfer, E. (2022) Short and long-term solubility, alkalizing effect, and thermal persistence of premixed calcium silicate-based sealers: AH Plus Bioceramic Sealer vs. Total Fill BC Sealer. *Materials*, 15, 7320.

Elsayed, M.A., Hassanien, E.E. & Elgendy, A.A.E. (2021) Ageing of TotalFill BC Sealer and MTA Fillapex in simulated body fluid. *European Endodontic Journal*, 6, 183–188.

Estrela, C., Carmo Souza, P.O., Barbosa, M.G., Aburad de Carvalhosa, A., Batista, A.C., Pinto Júnior, D.D.S. et al. (2019) Mesenchymal stem cell marker expression in periapical abscess. *Journal of Endodontics*, 45, 716–723.

Feng, Z., Jin, M., Liang, J., Kang, J., Yang, H., Guo, S. et al. (2023) Insight into the effect of biomaterials on osteogenic differentiation of mesenchymal stem cells: a review from a mitochondrial perspective. *Acta Biomaterialia*, 164, 1–14.

Forni, M., Bernardini, C., Zamparini, F., Zannoni, A., Salaroli, R., Ventrella, D. et al. (2020) Vascular Wall-mesenchymal stem cells differentiation on 3D biodegradable highly porous CaSi-DCPD doped poly (α-hydroxy) acids scaffolds for bone regeneration. *Nanomaterials*, 10, 243.

Gandolfi, M.G., Ciapetti, G., Taddei, P., Perut, F., Tinti, A., Cardoso, M.V. et al. (2010) Apatite formation on bioactive calcium-silicate cements for dentistry affects surface topography and human marrow stromal cells proliferation. *Dental Materials*, 26, 974–992.

Gandolfi, M.G., Parrilli, A.P., Fini, M., Prati, C. & Dummer, P.M. (2013) 3D micro-CT analysis of the interface voids associated with Thermafil root fillings used with AH plus or a flowable MTA sealer. *International Endodontic Journal*, 46, 253–263.

Gandolfi, M.G., Perut, F., Ciapetti, G., Mongiorgi, R. & Prati, C. (2008) New Portland cement-based materials for endodontics mixed with articaine solution: a study of cellular response. *Journal of Endodontics*, 34, 39–44.

Gandolfi, M.G., Shah, S.N., Feng, R., Prati, C. & Akintoye, S.O. (2011) Biomimetic calcium-silicate cements support differentiation of human orofacial mesenchymal stem cells. *Journal of Endodontics*, 37, 1102–1108.

Gandolfi, M.G., Taddei, P., Modena, E., Siboni, F. & Prati, C. (2013) Biointeractivity-related versus chemi/physisorption-related apatite precursor-forming ability of current root end filling materials. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 101, 1107–1123.

Gandolfi, M.G., Taddei, P., Siboni, F., Modena, E., Ciapetti, G. & Prati, C. (2011) Development of the foremost light-curable calcium-silicate MTA cement as root-end in oral surgery. Chemical-physical properties, bioactivity and biological behavior. *Dental Materials*, 27, 134–157, e134, e157.

García, C.C., Sempere, F.V., Diago, M.P. & Bowen, E.M. (2007) The post-endodontic periapical lesion: histologic and etiopathogenic aspects. *Medicina Oral, Patología Oral y Cirugía Bucal*, 12, 585–590.

Gautam, S., Thapa, A., Joshi, R. & Joshi, B. (2022) Outcome of root canal treatment using bioceramic sealer and resin based sealer: an observational analytical study. *Nepal Medical College Journal*, 24, 68–74.

Giacomino, C.M., Wealleans, J.A., Kuhn, N. & Diogenes, A. (2019) Comparative biocompatibility and osteogenic potential of two bioceramic sealers. *Journal of Endodontics*, 45, 51–56.

Hong, M.H., Lee, J.H., Jung, H.S., Shin, H. & Shin, H. (2022) Biomineralization of bone tissue: calcium phosphate-based inorganics in collagen fibrillar organic matrices. *Biomaterials Research*, 26, 42.

Howson, K.M., Aplin, A.C., Gelati, M., Alessandri, G., Parati, E.A. & Nicosia, R.F. (2005) The postnatal rat aorta contains pericyte progenitor cells that form spheroidal colonies in suspension culture. *American Journal of Physiology. Cell Physiology*, 289, 1396–1407.

Huang, W., Yang, S., Shao, J. & Li, Y.P. (2007) Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Frontiers in Bioscience*, 12, 3068–3092.

Invernici, G., Emanueli, C., Madeddu, P., Cristini, S., Gadau, S., Benetti, A. et al. (2007) Human fetal aorta contains vascular progenitor cells capable of inducing vasculogenesis, angiogenesis, and myogenesis in vitro and in a murine model of peripheral ischemia. *The American Journal of Pathology*, 170, 1879–1892.

Iurlaro, M., Scatena, M., Zhu, W.H., Fogel, E., Wieting, S.L. & Nicosia, R.F. (2003) Rat aorta-derived mural precursor cells express the Tie2 receptor and respond directly to stimulation by angiopoietins. *Journal of Cell Science*, 116, 3635–3643.

Jung, G.Y., Park, Y.J. & Han, J.S. (2010) Effects of HA released calcium ion on osteoblast differentiation. *Journal of Materials Science. Materials in Medicine*, 21, 1649–1654.

Jung, S., Sielker, S., Hanisch, M.R., Libricht, V., Schäfer, E. & Dammaschke, T. (2018) Cytotoxic effects of four different root canal sealers on human osteoblasts. *PLoS One*, 13, 0194467.

Karamifar, K., Tondari, A. & Saghiri, M.A. (2020) Endodontic periapical lesion: an overview on the etiology, diagnosis and current treatment modalities. *European Endodontic Journal*, 5, 54–67.

Kharouf, N., Arntz, Y., Eid, A., Zghal, J., Sauro, S., Haikel, Y. et al. (2020) Physicochemical and antibacterial properties of novel, premixed calcium silicate-based sealer compared to powder-liquid bioceramic sealer. *Journal of Clinical Medicine*, 9(10), 3096.

Koutroulis, A., Kuehne, S.A., Cooper, P.R. & Camilleri, J. (2019) The role of calcium ion release on biocompatibility and antimicrobial properties of hydraulic cements. *Scientific Reports*, 13(9), 19019.

Lee, J.K., Kwak, S.W., Ha, J.H., Lee, W. & Kim, H.C. (2017) Physicochemical properties of epoxy resin-based and bioceramic-based root canal sealers. *Bioinorganic Chemistry and Applications*, 2017, 2582849.

Lee, S.H., Oh, S., Al-Ghamdi, A.S., Mandorah, A.O., Kum, K.Y. & Chang, S.W. (2020) Sealing ability of AH Plus and GuttaFlow Bioseal. *Bioinorganic Chemistry and Applications*, 2020, 8892561.

Leonardi, R., Caltabiano, M., Pagano, M., Pezzuto, V., Loreto, C. & Palestro, G. (2003) Detection of vascular endothelial growth factor/ vascular permeability factor in periapical lesions. *Journal of Endodontics*, 29, 180–183.

Liao, J., Al Shahrani, M., Al-Habib, M., Tanaka, T. & Huang, G.T. (2011) Cells isolated from inflamed periapical tissue express mesenchymal stem cell markers and are highly osteogenic. *Journal of Endodontics*, 37, 1217–1224.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using realtime quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods*, 25, 402–408.

López-García, S., Myong-Hyun, B., Lozano, A., García-Bernal, D., Forner, L., Llena, C. et al. (2020) Cytocompatibility, bioactivity potential, and ion release of three premixed calcium silicate-based sealers. *Clinical Oral Investigations*, 24, 1749–1759.

Lozano-Guillén, A., López-García, S., Rodríguez-Lozano, F.J., Sanz, J.L., Lozano, A., Llena, C. et al. (2022) Comparative cytocompatibility of the new calcium silicate-based cement NeoPutty versus NeoMTA Plus and MTA on human dental pulp cells: an in vitro study. *Clinical Oral Investigations*, 26, 7219–7228.

Marrelli, M., Paduano, F. & Tatullo, M. (2013) Cells isolated from human periapical cysts express mesenchymal stem cell-like properties. *International Journal of Biological Sciences*, 9, 1070–1078.

Moinzadeh, A.T., Aznar Portoles, C., Schembri Wismayer, P. & Camilleri, J. (2016) Bioactivity potential of EndoSequence BC RRM putty. *Journal of Endodontics*, 42, 615–621.

Nagendrababu, V., Murray, P.E., Ordinola-Zapata, R., Peters, O.A., Rôças, I.N., Siqueira, J.F., Jr. et al. (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontology: a consensus-based development. *International Endodontic Journal*, 54, 1482–1490. Available from: https://doi.org/10.1111/iej.13542

Nair, P.N. (2006) On the causes of persistent apical periodontitis: a review. *International Endodontic Journal*, 39, 249–281.

Negri, S., Wang, Y., Sono, T., Lee, S., Hsu, G.C., Xu, J. et al. (2020) Human perivascular stem cells prevent bone graft resorption in osteoporotic contexts by inhibiting osteoclast formation. *Stem Cells Translational Medicine*, 9, 1617–1630.

Niu, L.N., Jiao, K., Wang, T.D., Zhang, W., Camilleri, J., Bergeron, B.E. et al. (2014) A review of the bioactivity of hydraulic calcium silicate cements. *Journal of Dentistry*, 42, 517–533.

Pasanisi, E., Ciavarella, C., Valente, S., Ricci, F. & Pasquinelli, G. (2019) Differentiation and plasticity of human vascular wall mesenchymal stem cells, dermal fibroblasts and myofibroblasts: a critical comparison including ultrastructural evaluation of osteogenic potential. *Ultrastructural Pathology*, 43, 261–272.

Pontoriero, D.I.K., Ferrari Cagidiaco, E., Maccagnola, V., Manfredini, D. & Ferrari, M. (2023) Outcomes of endodontic-treated teeth obturated with bioceramic sealers in combination with warm Gutta-Percha Obturation techniques: a prospective clinical study. *Journal of Clinical Medicine*, 12, 2867.

Primus, C., Gutmann, J.L., Tay, F.R. & Fuks, A.B. (2022) Calcium silicate and calcium aluminate cements for dentistry reviewed. *Journal of the American Ceramic Society*, 105, 1841–1863.

Primus, C.M., Tay, F.R. & Niu, L.N. (2019) Bioactive tri/dicalcium silicate cements for treatment of pulpal and periapical tissues. *Acta Biomaterialia*, 15(96), 35–54.

Rathinam, E., Govindarajan, S., Rajasekharan, S., Declercq, H., Elewaut, D., De Coster, P. et al. (2021) The calcium dynamics of human dental pulp stem cells stimulated with tricalcium silicate-based cements determine their differentiation and mineralization outcome. *Scientific Reports*, 11, 645.

Ricucci, D. & Siqueira, J.F., Jr. (2010) Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. *Journal of Endodontics*, 36, 1277–1288.

Ricucci, D., Loghin, S., Gonçalves, L.S., Rôças, I.N. & Siqueira, J.F., Jr. (2018) Histobacteriologic conditions of the apical root canal system and periapical tissues in teeth associated with sinus tracts. *Journal of Endodontics*, 44, 405–413.

Santiago, M.C., Gomes-Cornélio, A.L., de Oliveira, L.A., Tanomaru-Filho, M. & Salles, L.P. (2021) Calcium silicate-based cements cause environmental stiffness and show diverse potential to induce osteogenesis in human osteoblastic cells. *Scientific Reports*, 11, 16784.

Sanz, J.L., López-García, S., Rodríguez-Lozano, F.J., Melo, M., Lozano, A., Llena, C. et al. (2022) Cytocompatibility and bioactive potential of AH Plus bioceramic sealer: an in vitro study. *International Endodontic Journal*, 55, 1066–1080.

Schröder, H.C., Wang, X.H., Wiens, M., Diehl-Seifert, B., Kropf, K., Schloßmacher, U. et al. (2012) Silicate modulates the cross-talk between osteoblasts (SaOS-2) and osteoclasts (RAW 264.7 cells): inhibition of osteoclast growth and differentiation. *Journal of Cellular Biochemistry*, 113, 3197–3206.

Shokouhinejad, N., Nekoofar, M.H., Razmi, H., Sajadi, S., Davies, T.E., Saghiri, M.A. et al. (2012) Bioactivity of EndoSequence root repair material and bioaggregate. *International Endodontic Journal*, 45, 1127–1134.

Shokrzadeh, M., Motafeghi, F.S., Lotfizadeh, A., Ghorbani, M. & Haddadi, K.A. (2023) Cytotoxicity and mineralization activity of calcium silicate-based root canal sealers compared to conventional resin-based sealer in human gingival fibroblast cells. *International Journal of Dentistry*, 2023, 4376579.

Siboni, F., Taddei, P., Zamparini, F., Prati, C. & Gandolfi, M.G. (2017) Properties of BioRoot RCS, a tricalcium silicate endodontic sealer modified with povidone and polycarboxylate. *International Endodontic Journal*, 50, 120–136.

Song, M., Park, M.G., Kwak, S.W., Kim, R.H., Ha, J.H. & Kim, H.C. (2022) Pilot evaluation of sealer-based root canal obturation using epoxy-resin-based and calcium-silicate-based sealers: a randomized clinical trial. *Materials*, 15, 5146. Available from: https://doi.org/10.3390/ma15155146

Souza, L.C., Neves, G.S.T., Kirkpatrick, T., Letra, A. & Silva, R. (2023) Physicochemical and biological properties of AH Plus bioceramic. *Journal of Endodontics*, 49, 69–76.

Spencer, G.J., Utting, J.C., Etheridge, S.L., Arnett, T.R. & Genever, P.G. (2006) Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis in vitro. *Journal of Cell Science*, 119, 1283–1296.

Spinelli, A., Zamparini, F., Lenzi, J., Gandolfi, M.G. & Prati, C. (2023) Clinical evaluation of a novel premixed tricalcium

silicate containing bioceramic sealer used with warm carrier-based technique: a 12-month prospective pilot study. *Applied Sciences*, 13, 11835.

Sun, J., Wei, L., Liu, X., Li, J., Li, B., Wang, G. et al. (2009) Influences of ionic dissolution products of dicalcium silicate coating on osteoblastic proliferation, differentiation and gene expression. *Acta Biomaterialia*, 5, 1284–1293.

Taddei, P., Tinti, A., Gandolfi, M.G. & RossiP, P.C. (2009) Vibrational study on the bioactivity of Portland cement-based materials for endodontic use. *Journal of Molecular Structure*, 924–926, 548–554.

Tay, F.R., Pashley, D.H., Rueggeberg, F.A., Loushine, R.J. & Weller, R.N. (2007) Calcium phosphate phase transformation produced by the interaction of the Portland cement component of white mineral trioxide aggregate with a phosphate-containing fluid. *Journal of Endodontics*, 33, 1347–1351.

Tibúrcio-Machado, C.S., Michelon, C., Zanatta, F.B., Gomes, M.S., Marin, J.A. & Bier, C.A. (2021) The global prevalence of apical periodontitis: a systematic review and meta-analysis. *International Endodontic Journal*, 54, 712–735.

Turner, J., Nandakumar, A., Anilbhai, N., Boccaccini, A.R., Jones, J.R. & Jell, G. (2023) The effect of Si species released from bioactive glasses on cell behaviour: a quantitative review. *Acta Biomaterialia*, 170, 39–52.

Virtej, A., Løes, S.S., Berggreen, E. & Bletsa, A. (2013) Localization and signaling patterns of vascular endothelial growth factors and receptors in human periapical lesions. *Journal of Endodontics*, 39, 605–611.

Viti, F., Landini, M., Mezzelani, A., Petecchia, L., Milanesi, L. & Scaglione, S. (2016) Osteogenic differentiation of MSC through calcium signaling activation: transcriptomics and functional analysis. *PLoS One*, 11, e0148173.

Wang, K.X. & Denhardt, D.T. (2008) Osteopontin: role in immune regulation and stress responses. *Cytokine & Growth Factor Reviews*, 19, 333–345.

Wang, P., Zhao, L., Liu, J., Weir, M.D., Zhou, X. & Xu, H.H. (2014) Bone tissue engineering via nanostructured calcium phosphate biomaterials and stem cells. *Bone Research*, 2, 14017.

Xue, K., Hu, G., Wu, L., Han, H., Sun, Y., Gan, K. et al. (2023) The bioceramic sealer iRoot SP promotes osteogenic differentiation of human stem cells from apical papilla via miR-141-3p/SPAG9/MAPK signalling pathway. *International Endodontic Journal*, 56, 1241–1253.

Zamparini, F., Prati, C., Taddei, P., Spinelli, A., Di Foggia, M. & Gandolfi, M.G. (2022) Chemical-physical properties and bioactivity of new premixed calcium silicate-bioceramic root canal sealers. *International Journal of Molecular Sciences*, 23, 13914.

Zamparini, F., Siboni, F., Prati, C., Taddei, P. & Gandolfi, M.G. (2019) Properties of calcium silicate-monobasic calcium phosphate materials for endodontics containing tantalum pentoxide and zirconium oxide. *Clinical Oral Investigations*, 23, 445–457.

Zamparini, F., Spinelli, A., Cardinali, F., Ausiello, P., Gandolfi, M.G. & Prati, C. (2023) The use of premixed calcium silicate bioceramic sealer with warm carrier-based technique: a 2-year study for patients treated in a master program. *Journal of Functional Biomaterials*, 14, 164.

Zaniboni, A., Bernardini, C., Alessandri, M., Mangano, C., Zannoni, A., Bianchi, F. et al. (2014) Cells derived from porcine aorta tunica media show mesenchymal stromal-like cell properties in in vitro culture. *American Journal of Physiology. Cell Physiology*, 306, 322–333.

Zaniboni, A., Bernardini, C., Bertocchi, M., Zannoni, A., Bianchi, F., Avallone, G. et al. (2015) In vitro differentiation of porcine aortic vascular precursor cells to endothelial and vascular smooth muscle cells. *American Journal of Physiology. Cell Physiology*, 309, 320–331.

Zou, S., Ireland, D., Brooks, R.A., Rushton, N. & Best, S. (2009) The effects of silicate ions on human osteoblast adhesion, proliferation, and differentiation. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 90, 123–130.

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