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Original Article

Ex-vivo evaluation of intratubular antibacterial activity and biocompatibility of two hydraulic calcium silicate-based endodontic sealers

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Abstract

Purpose: This study evaluated the antibacterial activity of two hydraulic calcium silicate-based sealers, NeoSealer Flo and AH Plus Bioceramic, against *Enterococcus faecalis* (*E. faecalis*) biofilm and their biocompatibility with pre-osteoblastic cells.

Methods: Thirty-one extracted human lower incisors with single canals were inoculated with *E. faecalis*, and their root canals were subjected to three types of filling: A) NeoSealer Flo (n = 10), B) AH Plus Bioceramic (n = 10), and C) positive control (n = 10), as well as one negative control. The teeth were embedded in resin and sectioned at their coronal, middle, and apical thirds, yielding 160 sections. Bacterial viability was assessed by confocal laser scanning microscopy using LIVE/DEAD staining. Biocompatibility was evaluated using the bicinchoninic acid protein assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Results: NeoSealer Flo and AH Plus Bioceramic showed comparable antibacterial activity. In the coronal third of the tooth, viable bacteria were estimated at 44.1 ± 4.0% and 43.5 ± 4.0%, respectively, versus 85.7 ± 5.2% in controls. Similar results were obtained in the middle and apical thirds. Viability was significantly higher in the coronal third ($P < 0.05$). No significant differences in cell number or protein expression were observed between the sealers and the controls ($P > 0.05$).

Conclusion: Both sealers demonstrated similar antibacterial effectiveness and biocompatibility, with greater bacterial viability in the coronal region.

Keywords: antibacterial, bioceramic sealer, biocompatibility, *Enterococcus faecalis*, hydraulic calcium silicate-based sealers, root canal treatment

Introduction

The long-term success of endodontic treatment primarily depends on chemo-mechanical disinfection of the root canal system and the stability of both the coronal restoration and the apical seal [1]. While chemo-mechanical procedures for root canals aim to reduce the bacterial load, they are unable to completely eliminate all bacteria from the endodontic environment [2,3]. The persistence of microorganisms within the root canal system is a major cause of endodontic failure [4]. *Enterococcus faecalis* (*E. faecalis*) is a gram-positive bacterium frequently associated with persistent infections and post-treatment apical periodontitis [5]. Its biofilm represents a significant challenge in endodontics because of its strong adhesion to tooth surfaces and ability to resist standard chemo-mechanical disinfection procedures [6]. Given these challenges, root canal obturation and coronal restorative procedures are essential for achieving the sequestration of bacteria and preventing their proliferation [5]. Inadequate sealing of the root canal system remains one of the most common causes of endodontic failure [7]. Consequently, the choice of appropriate obturation material is central to the long-term success of root canal treatment. A variety of obturation materials are currently used in clinical practice, including zinc oxide, eugenol, calcium hydroxide, resin-based sealants, glass ionomers, and hydraulic sealers [8-10]. Hydraulic sealers, also known as calcium silicate-based sealants, set in the presence of water or moisture and have been introduced as possible alternatives to traditional endodontic sealers. Modern hydraulic calcium silicate-based sealers, commonly referred to as bioceramics, have shown promising results in terms of seal quality, physico-chemical properties, and antibacterial activity [9,11,12]. Obturation materials with antimicrobial properties can contribute to the elimination of residual micro-organisms within the root canal system and limit the reinfiltration of bacteria from the oral cavity, thus improving the long-term outcome of endodontic treatment [10]. Recently, these sealers have shown promising preliminary results in terms of bioactivity and biocompatibility [13]. Once bacteria have been eliminated, healing of apical periodontitis requires remodeling of the granulomatous tissue and proliferation of osteoblast precursors into mature osteoblasts [14]. The bioactivity and biocompatibility of these materials may be explained by their alkaline pH and ability to release calcium ions that support apical tissue remineralization [15]. These features are crucial and ensure successful therapy, preventing possible complications due to unintended apical extrusion during endodontic procedures [16].

Data regarding the antibacterial properties of hydraulic calcium silicate-based sealers are still scarce and controversial. Therefore, the primary aim of the present study was to evaluate and compare the antibacterial activity of two common bioactive endodontic cements against *E. faecalis* biofilm and assess the biocompatibility of these sealers with osteoblastic lineage cells.

Materials and Methods

The present ex vivo study was conducted according to The Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) guidelines [17] (Fig. 1) and was approved by the Local Ethics Committee of the Region of Tuscany South-Eastern Area (Protocol no.7/2021); informed consent was obtained from all patients before tooth extraction.

Sample size calculation

Sample size calculation was performed using G*Power software (version 3.1.9.4; Heinrich Heine University, Düsseldorf, Germany) based on the methodological approach adopted in previous peer-reviewed studies using similar experimental models [18-20]. Given the substantial differences reported, an effect size (f = 0.6) was estimated from the data reported in these studies. Using one-way ANOVA with a significance level (α) of 0.05 and a statistical power of 80%, the required total sample size was calculated to be 10 per group. This sample size was deemed sufficient for detection of a moderate effect size with acceptable power.

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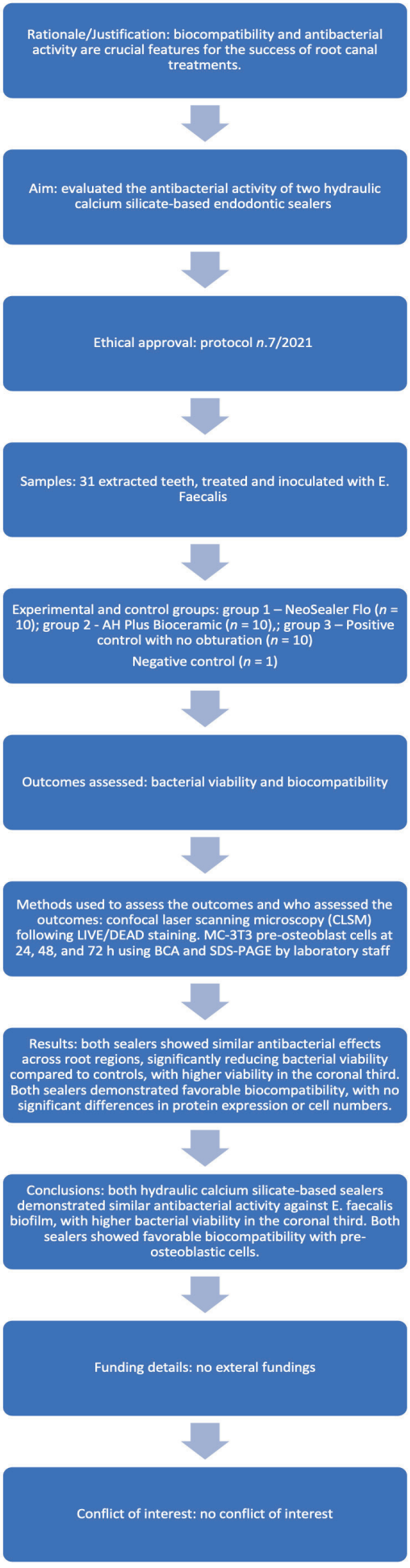

Fig. 1 PRILE flow chart

Sample selection and preparation

Thirty-one freshly extracted human lower incisors, with a mature root apex and a single straight canal, were selected and stored in 0.1% thymol solution at 4°C until use. The reason for extraction was periodontal disease. Exclusion criteria included caries, root resorption, fractures, fillings, previous endodontic treatment, multiple canals, immature apices, calcifications, or curvature. Preoperative radiographs ensured eligibility. Teeth were cleaned with a periodontal curette (Hu-Friedy, Chicago, IL, USA) and decoronated using a water-cooled diamond disk (KG Scorensen, Barueri, SP, Brazil) to obtain roots 12 mm in length.

Before infection, apical patency was confirmed with a 10-K file (Dentsply Maillefer, Ballaigues, Switzerland). Root canals were prepared using WaveOne Gold (Dentsply Maillefer) glider (015; 02), small (020; 02), primary (025; 07), and medium (035; 06) files until working length and irrigated with 5.25% NaOCl after each file had been changed. Samples were placed in an ultrasonic bath for 10 min in 17% EDTA, and then in 5.25% NaOCl to open the dentinal tubules (Ferraz et al., 2001). Root canals were rinsed with distilled water and then autoclaved at 121°C for 20 min. Specimens were individually placed in Eppendorf tubes with 1.5 mL of sterile brain heart infusion broth (BHI) and incubated at 37°C for 24 h to confirm sterility.

Bacterial culture and growth

The post-endodontic treatment isolate of *E. faecalis* was maintained in a tryptic soy broth (TSB) agar plate at 37°C. An overnight preculture was obtained by picking a single colony of post- endodontic clinical isolate (PE) *E. faecalis* streaked in sterile TSB medium and incubated overnight at 37°C with agitation at 220 rpm. Then, bacteria from the overnight preculture were diluted 1:100 in TSB medium and grown at 37°C with agitation at 220 rpm to reach an optical density (O.D.) of 1 measured with a spectrophotometer at a wavelength of 600 nm (Jenway 6305 UV/Visible, Antylia Scientfic, Vernon Hill, IL, USA). After reaching the final O.D., the bacterial suspension was centrifuged at 4,000 rpm for 10 min. The resulting pellet was resuspended in fresh TSB medium to obtain a final concentration of 108 colony-forming units (CFU)/mL bacteria.

Bacterial infection of tubules

After positioning each tooth in a 1.5-mL tube to keep it upright, 30 µL of the final bacterial suspension was inoculated into the root canal of thirty teeth, while only TSB medium was placed in one single tooth that served as a negative control. Then, all the tubes containing the teeth were isolated with parafilm, immersed in water, and sonicated at 37 kHz for 5 min (Elma P30H, Elmasonic P Ultrasonic Cleaner, Singen, Germany). Successively, all the teeth were covered with 500 µL of TSB medium to allow biofilm formation within each root canal. Then, the tubes were isolated with parafilm to simulate anaerobic conditions and incubated at 37°C for 1 week. In the meantime, serial 10^{-1} to 10^{-14} dilutions of the inoculated bacterial suspension were prepared and plated in TSB-agar plates to assess the presence and concentration of the bacteria within the root canals. CFU were counted after incubation of the plates overnight at 35°C. After 1 week of incubation, the medium and the supernatant within the root canals were removed.

Canal obturation

Sample teeth were filled with the hydraulic calcium silicate-based sealers (Table 1) according to the following randomization: Group A (n = 10) was filled with NeoSealer Flo (Avalon Biomed, Houston, TA, USA) and Group B (n = 10) was filled with AH Plus Bioceramic sealer (Dentsply Sirona, Charlotte, NC, USA). NeoSealer Flo is a premixed hydraulic calcium silicate-based sealer containing the following bioactive components: tricalcium silicate (<25%), calcium aluminate (<25%), dicalcium silicate (<10%), grossite (<6%) and tricalcium aluminate (<5%). Approximately 50% of the formulation is tantalite, which is radiopaque. Trace amounts of calcium sulfate have also been reported (<1%). AH Plus Bioceramic is a premixed hydraulic calcium silicate-based sealant consisting primarily of zirconium dioxide as the radiopaque agent (50-70%) and tricalcium silicate (10-15%) as the bioactive component. The manufacturer also specifies the presence of dimethyl sulfoxide (DMSO) and traces of lithium carbonate and thickening agents [12,21,22]. Both sealers were delivered using a dedicated tip, i.e., the Flex Flo Tip (Avalon Biomed), by inserting the tip

Table 1 Chemical characteristics of the experimental products

Product name (manufacturer, city, country)	Chemical matrix	Presentation	Composition
NeoSelaer Flo (Avalon Biomed, Houston, TA, USA)	hydraulic calcium silicate-based endodontic sealer	premixed paste	tricalcium silicate (<25%), calcium aluminate (<25%), dicalcium silicate (<10%), grossite (<6%), tricalcium aluminate (<5%), tantalite (50%), calcium sulfate (<1%)
AH Plus Bioceramic (Dentsply Sirona, Charlotte, NC, USA)	hydraulic calcium silicate-based endodontic sealer	premixed paste	zirconium dioxide (50-70%), tricalcium silicate (10-15%), dimethyl sulfoxide (DMSO), lithium carbonate, Thickening agents

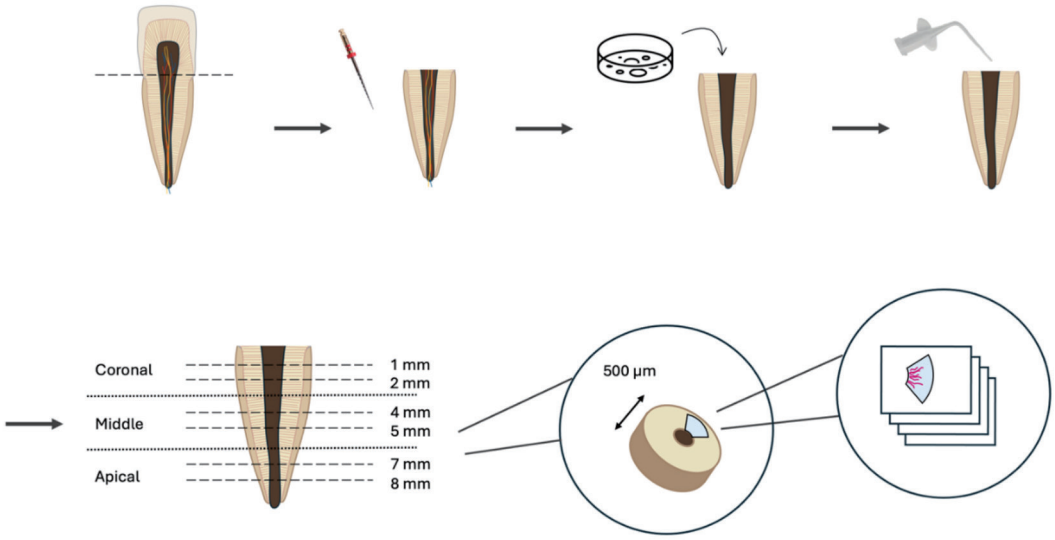

Fig. 2 Schematic illustration of the experimental procedure used for evaluating the intratubular antibacterial activity of the hydraulic calcium silicate-based sealers

at the coronal level and applying pressure to the dispenser until the sealer exited the apical foramen. Group C (n = 10) served as a positive control that was left unfilled and merely infected with the bacterial culture, while another tooth was merely inoculated with culture medium lacking bacteria as a negative control.

All samples were incubated at 37°C with 100% humidity for 10 days to allow the sealer to set.

Sample preparation for confocal laser scanning microscopy

The dentin specimens were embedded in wax (Kerr Utility Wax Strips) using cylindrical containers with a diameter of 15 mm and a height of 40 mm, which were filled with 3 mm of clear epoxy resin (Hardrock 554, Phase 20, Fig. 2). At 48 h when the resin polymerization was complete, the embedded samples were removed from the containers and sectioned transversely with a thickness of 500 µm at 6 points – coronal third (1 mm and 2 mm); middle third (7 mm and 8 mm); apical third (11 mm and 12 mm) – using a Leica SP1600 microtome under a continuous flow of water. One tooth in the positive control group was damaged during sectioning and was therefore excluded from the analysis. In total, 60 sections were collected from each hydraulic calcium silicate-based group, 60 sections from the positive control group and 6 sections from the negative control tooth, making a total of 180 sections. The total number of samples was 60 for the coronal third, 60 for the middle third, and 60 for the apical third. The sections were labeled and placed in multiwell plates. Bacterial viability was assessed using the LIVE/DEAD assay (Molecular Probes LIVE/DEAD BacLight, Invitrogen, Waltham, MA, USA). The staining phase was carried out by immersion in a solution containing 1.67 mM SYTO 9 and 18.7 mM propidium iodide for 30 min at 37°C in the dark. Subsequently, the sections were washed with phosphate-buffered saline (PBS) and maintained at 4°C in PBS until completion of image acquisition. The staining solution consisted of SYTO 9 at a final concentration of 5 µM and propidium iodide at 30 µM, and specimens were incubated in the dark for 15 min at room temperature prior to imaging.

Images of each section were acquired at ×20 magnification using a Leica SP8 AOBS laser confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a white light laser. The samples were exposed to the laser at different wavelengths to evaluate the red and green fluorescence resulting from LIVE/DEAD staining. Specifically, excitation at a wavelength of 488 nm was used to visualize green fluorescence with a signal intensity of 20% and a signal detection band between 494 nm and 535 nm. Red fluorescence was visualized by excitation at a wavelength of 546 nm, with a signal intensity of 41.8% and a signal detection band between 592 nm and 636 nm. Each image was obtained by acquiring a variable number of images in the Z plane with a Z stack of 1 µm. All images showing a green or red fluorescence signal were acquired.

Figure 2 shows a schematic image illustrating the experimental procedures applied.

Image analysis

Confocal laser scanning microscopy (CLSM) images were then processed and analyzed using Fiji software (National Institutes of Health, Bethesda, MD, USA).

The following procedure was used for morphometric evaluation of each section. The “hyperstack” option was selected for pixel visualization. To display all sections on the overlapping Z-plane, the “max intensity” projection type was selected and the image containing all sections on the Z-plane was displayed. The “split channels” function was then used to obtain the green and red fluorescence images separately. To evaluate the two fluorescence colors together, the “merge” function was used, as it is useful for qualitative evaluation. Finally, the “mosaic” function was used to visualize the entire section in the X, Y, and Z planes.

Data extraction was performed using a set of features within the Fiji software. Specifically, a section was taken from the negative (no bacteria) control sample, since the signal was derived from the autofluorescence of dentin or the non-specific fluorescence of the LIVE/DEAD kit dyes. The “threshold” function was then applied, allowing the signal power to be reduced until autofluorescence and non-specific fluorescence signals were eliminated. This yielded a value of 7.73%, which was used for standardization by applying it via the “threshold” function to each subsequent section. Then, the “polygon selection” function was used to remove the signal from the root canal as well as any artifacts on the section. After obtaining the artifact-free image in both the red and green fluorescence modes, the

"create selection" function was used, allowing quantification of the signal. After obtaining the value of the area occupied by the signal, the percentage of the signal resulting from red and green fluorescence was calculated using the following formula:

$$\%\text{Green fluorescence} = \frac{\text{Green fluorescence area}}{\text{Green fluorescence area} + \text{Red fluorescence area}} \times 100$$

$$\%\text{Red fluorescence} = 100 - \%\text{Green fluorescence}$$

The percentage green and red fluorescence values for each section were recorded on an Excel datasheet (Microsoft Corp., Redmond, WA, USA) for statistical analysis.

Cell culture and biocompatibility assay

A murine osteoblast precursor cell line (MC3T3-Subclone 14) (ATCC, Boston, MA, USA) was cultured according to a previously established protocol in alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Briefly, cells were cultured at 37°C in 5% CO_2 in media composed of alpha-modified minimum essential medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA) and 1% glutamine, penicillin and streptomycin solution (Gemini Bio-Products, West Sacramento, CA, USA).

From the cell culture in Petri dishes, multiwell plates were set up. Each sample was seeded in duplicate for a total of two wells per sample per plate. Each plate was seeded with 80,000 cells per well. Twenty-four hours after seeding, the hydraulic calcium silicate-based sealer and the cells were brought into contact using transwell devices. An equal amount of sealer by weight was deposited in direct contact with the entire area of the transwell device. Cells were harvested at 24, 48, and 72 h using standard procedures. After removal of the supernatant, the samples were washed once with PBS to remove residual medium and centrifuged again.

Cell viability (in terms of cell number) was assessed using trypan blue exclusion (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Significant differences (i.e., $P < 0.05$) between treatments were displayed as a bar chart.

The same samples were analyzed for protein expression. Cells were homogenized in a lysis buffer consisting of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and Mammalian Lysis Buffer (Promega, Madison, WI, USA) in a volume ratio of 1:500, added to the pellet for 30 min at 4°C in a refrigerator. The lysates were then centrifuged at 12,000 rpm for 10 min and the protein supernatant was stored at −20°C.

The protein quantification assay was performed using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific): the two components of the kit (Reagent A and Reagent B) were mixed in a 50:1 ratio. A 96-well plate was used and samples were loaded in triplicate with 200 µL of bicinchoninic acid (BCA) solution and 10 µL of the 5-fold-diluted sample.

After incubation at 37°C for 30 min, absorbance readings were taken at a wavelength of 571 nm using a Multiskan FC Microplate Photometer instrument (Thermo Fisher Scientific).

The results were compared with a calibration line previously obtained by performing the same procedure with a series of dilutions of known concentrations of bovine serum albumin (concentrations were calculated from 0 mg/ml BSA, then 5, 6, 8, 10, and 12 mg/ml). Data were recorded as protein content and total protein for each sealer sample.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue protein expression assay

For semi-quantitative analysis of protein expression, 40-µg protein extracts from each sample were loaded onto 7.5% polyacrylamide gels and stained with the generic dye Coomassie blue, which stains differently depending on whether it is in anionic or cationic form: upon binding to a protein, the negatively charged form of the dye stabilizes and produces a blue color, demonstrated as blue bands in the protein gel. Sample preparation was performed by mixing an appropriate volume of sample (corresponding to 20 µg protein) with a proportional amount of sample buffer (130 mM Tris-HCl, pH 6.8, 4% SDS, 50% [w/v] glycerol, 0.01% bromophenol blue) and dithiothreitol (DTT) which was added to the total volume in a 1:4 ratio.

Table 2 Mean and standard deviation of viable bacterial count (%)

	Group A (NeoSealer Flo) $n = 10$	Group B (AH Plus Bioceramic sealer) $n = 10$	Group C (positive control) $n = 10$
	mean ± SD	mean ± SD	mean ± SD
Coronal third	$44.1 \pm 4^{a,c}$	$43.5 \pm 4^{a,c}$	85.7 ± 5.2^{b}
Medium third	$41.8 \pm 4.6^{a,c}$	$40.8 \pm 3.1^{a,c}$	86.2 ± 4.9^{b}
Apical third	39.0 ± 2.8^{a}	38.9 ± 2.6^{a}	86.2 ± 4.9^{b}

Statistical analyses (ANOVA and Bonferroni *post hoc* tests) were performed using raw fluorescence data obtained from CLSM images. The values represent the percentage of green fluorescence (viable bacteria) relative to the total stained area and were calculated solely for illustrative and comparative purposes after statistical evaluation. Different superscript letters indicate a statistically significant difference ($P < 0.05$) between groups or among regions (ANOVA).

Each sample was then subjected to denaturation by heating at 100°C for 5 min. For each sample, 20 µg of protein was loaded into one of the gel wells. The electrophoretic run was performed using the Mini-PROTEAN Tetra System Bio-Rad cell (Bio-Rad, Hercules, CA, USA) at 100 mV for 1 h and 15 min.

At the end of the run, each gel was placed in an aqueous Coomassie blue dye solution, and rinsed with undistilled water to remove excess dye from the gels so that protein bands could be clearly identified.

Statistical analysis

Since the percentage values of red and green fluorescence obtained using morphometric analyses were complementary, statistical analysis was performed only for the green fluorescence data. The Shapiro-Wilk test was used to confirm the normal distribution of the data, and Levene's test was employed to evaluate the homogeneity of variances among groups. Both assumptions for parametric testing were satisfied ($P > 0.05$). Therefore, comparisons of mean green fluorescence among groups and across root regions (coronal, middle, and apical thirds) were conducted using one-way ANOVA, followed by Bonferroni *post hoc* tests to identify statistically significant differences. The same test was performed to compare the BCA protein assay at different times, while for comparison of data between hydraulic calcium silicate-based sealers, Student's *t* test was applied. The level of significance was set at $P < 0.05$. Data analysis was performed using Stata 11.2 (StataCorp LLC, College Station, TA, USA).

Results

Quantification of bacterial colonization in endodontically treated teeth obturated with hydraulic calcium silicate-based sealers

The mean and standard deviations of the viable bacteria percentages for each group are shown in Table 2. No significant differences ($P > 0.05$) in terms of bacterial viability were evident between groups A (NeoSealer Flo) and B (AH Plus Bioceramic sealer) for all root regions. However, a significant difference ($P < 0.05$) was found between groups A and B and the positive (C) control. As reported above, the negative control was used to subtract autofluorescence and no viable bacteria were present. There was also a significant difference ($P < 0.05$) in bacterial viability between the coronal and apical thirds both in groups A and B. Representative confocal microscopy images are shown in Fig. 3.

Biocompatibility of hydraulic calcium silicate-based sealers with the osteoblast MC-3T3 cell line

AHPlus Bioceramic and Neosealer Flo-treated MC3T3 osteoblast cells showed good tolerance against both biocercamics for 24, 48, and 72 h. The results, in terms of cell number counted using Trypan blue dye exclusion, are displayed as bar charts in Fig. 4. For both hydraulic calcium silicate-based sealers and relative controls, a statistically significant difference at $P < 0.05$ was evident for every treatment time (24, 48 or 72 h) relative to 0 h, and between 72 h and 24 h. Moreover, for NeoSealer Flo and in the control group for AHPlus Bioceramic, a significant difference was evident between 72 h and 48 h. Notably, at every testing point, there was no significant difference between the treated samples and control samples for either of the sealers.

With regard to the total amount of protein, the chart displayed in Fig. 5 demonstrates that there was no significant difference between cells treated with NeosealerFlo and the corresponding control at each time point. There

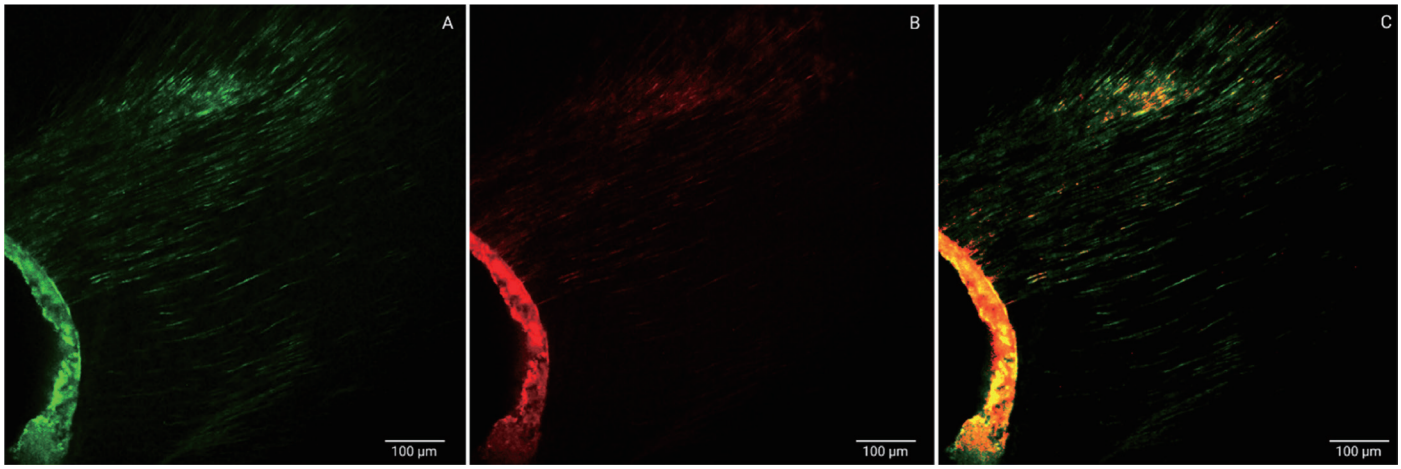

Fig. 3 Confocal laser scanning microscopy images in white light acquisition mode, showing intratubular dead and viable bacteria stained in the LIVE/DEAD assay in a transverse root section. A) Green acquisition representing intratubular viable bacteria. B) Red acquisition representing intratubular dead bacteria. (C) Both green/red acquisition of intratubular dead and viable bacteria.

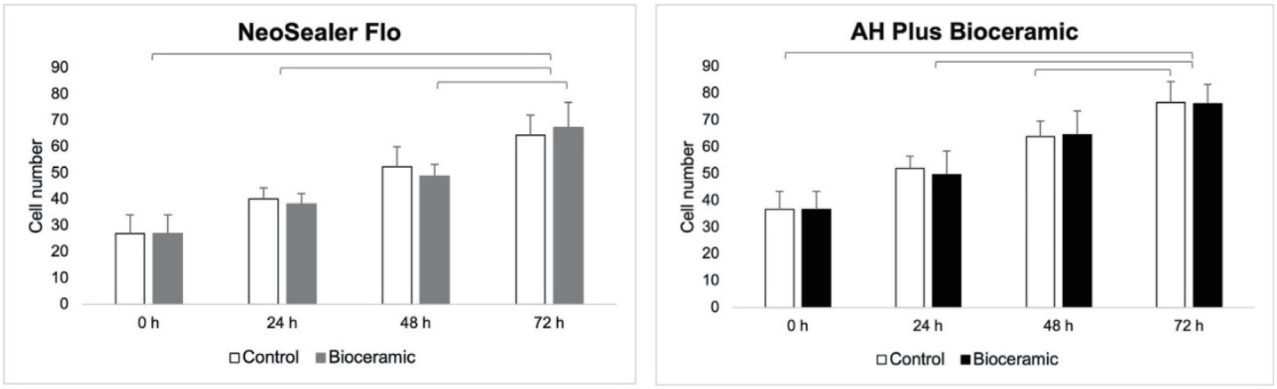

Fig. 4 Bar chart representing the cell count estimated by trypan blue exclusion

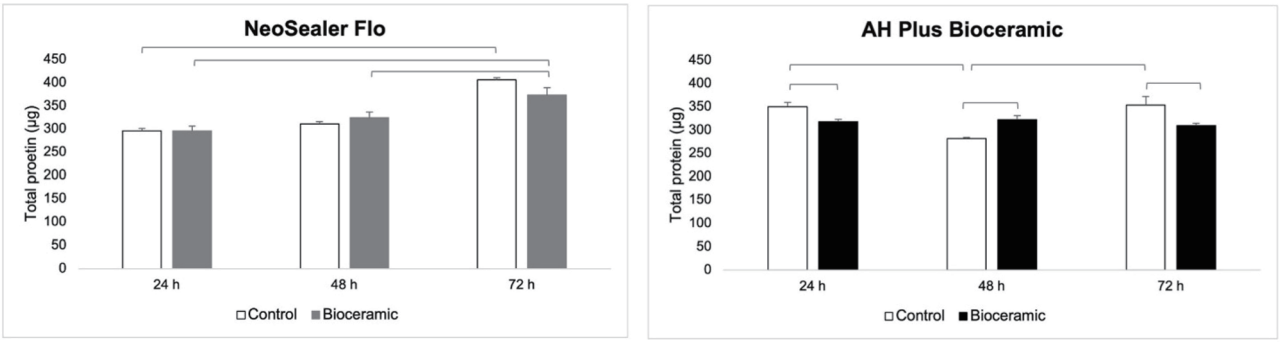

Fig. 5 Graphs representing the total amount of protein at different time points, estimated by the BCA quantitative assay for each of the two hydraulic calcium silicate-based sealers tested.

was a significant increase at 72 h in comparison to 24 h and 48 h in the treated group, and a significant increase at 72 h relative to 24 h in the control group.

For AH Plus Bioceramic, the graph shows that, at every time point examined, there was a significant difference in the total amount of protein between the treated and control groups ($P < 0.05$). Comparison of the treated groups at different time points revealed no discernible variation, but the protein level in the control group was significantly lower at 48 h than at 24 h or 72 h.

SDS-PAGE and Coomassie blue semi-quantitative protein expression assay

Figure 6 shows the polyacrylamide gels stained with Coomassie blue. There were no evident differences in protein expression either within the same sealer group or between the different sealers. In addition, protein expression in the samples placed in contact with the hydraulic calcium silicate-based sealers was similar to that in the relative control group, as reflected by the blue bands.

There were also no obvious differences in the staining intensity of the protein bands among cell groups cultured in contact with the sealers and the groups that served as controls.

Discussion

The antibacterial effect of endodontic sealers after setting is a desirable property [12,23]. The existing lack of standardized protocols means that data on the antibacterial properties of hydraulic sealers are scarce [24]. The aim of the present study was to assess the antibacterial efficacy of two recently developed pre-mixed hydraulic sealers, NeoSealer Flo and AH Plus Bioceramic Sealer, using CLSM with a previously validated method [25]. Both sealers reduced viable bacteria by nearly 50% relative to controls with no significant difference between them.

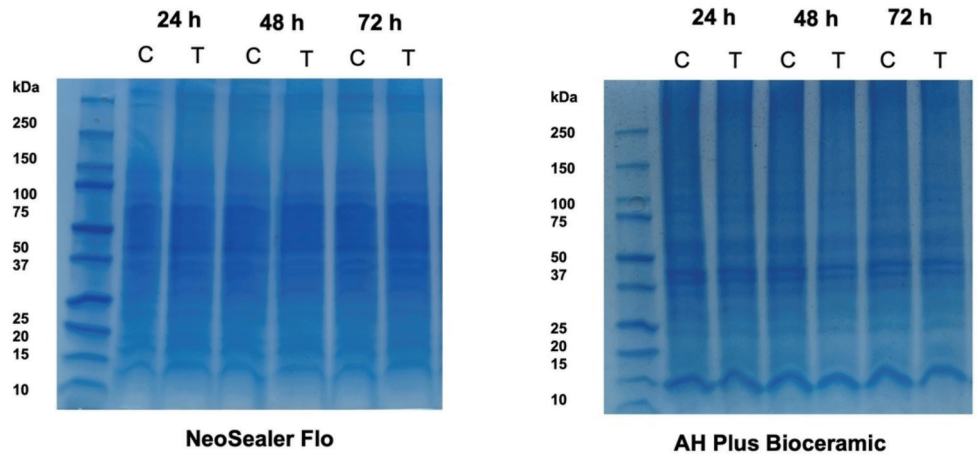

Fig. 6 Results of semi-quantitative SDS-PAGE protein expression assay using polyacrylamide gels stained with Coomassie blue (40 µg of protein loaded in each lane)

A recent study has reported similar findings, suggesting that NeoSealer Flo and AH Plus Bioceramic Sealer have comparable alkalizing activity and calcium release [11]. Subtle variations in their antibacterial effects may be due to differences in their compositions [11]. The hydraulic nature of these sealers may account for their antibacterial action [26]. Contact with dentin moisture triggers a hydration reaction, which results in the formation of calcium hydroxide and calcium silicate hydrogel, leading to an alkaline environment hostile to bacteria [21]. Recent studies demonstrating strong bactericidal effects of NeoSealer Flo [21] and AH Plus Bioceramic Sealer [15] support the present findings.

E. faecalis biofilm is commonly used to mimic endodontic infections [27,28]. While multispecies biofilms better replicate clinical scenarios [28], standardizing methodologies for management of competitive interactions remain challenging, favoring a monospecies biofilm model [29].

Unlike other methods, CLSM requires minimal sample preparation, preserves biofilm hydration and structure, and facilitates the quantification of both viable and viable but non-culturable bacteria [27]. Furthermore, it enables assessment of the LIVE/DEAD bacterial ratio [29] and biofilm distribution laterally within dentinal tubules [27] and longitudinally throughout the root [25]. Interestingly, the within-group comparison revealed a significantly higher percentage of viable bacteria in the coronal third relative to the apical third in groups A and B, probably due to reduced tubule density at the apex [25] and tubular sclerosis that begins at the apical level [30], reducing the penetration depth of the sealers.

In terms of the assessment of antibacterial properties, the present study had some limitations, such as the use of a mono-species biofilm model, a risk of bacterial displacement during sample preparation, and potential errors due to dentin debris and the smear layer affecting fluorochrome retention. Additionally, as an ex vivo study, the present investigation was unable to consider various clinical factors that might potentially influence antibacterial efficacy. Therefore, further research, including mixed-species biofilm models and clinical studies, will be necessary. Furthermore, the absence of a conventional sealer as a positive control may have limited direct comparisons with standard endodontic materials, and future studies will need to address this issue to broaden the clinical relevance.

In line with a previous CLSM-based study [25], which included six uninoculated roots as negative controls, the present study used a non-infected specimen to eliminate any background signal and calibrate the fluorescence thresholds. This type of methodological approach is essential for distinguishing true bacterial fluorescence from dentin autofluorescence or non-specific dye signals, ensuring the accuracy of viability assessments. Although a single negative control was sufficient for this purpose, future studies may benefit from the inclusion of additional negative or obturated controls to further validate the results of image analysis.

A secondary purpose of this study was to evaluate the biocompatibility of the tested hydraulic calcium silicate-based sealers to ensure the absence of any cytotoxicity accompanying the antibacterial effect. The biocompatibility analysis yielded promising results, in agreement with the relevant literature, confirming that the two sealers tested had good biocompatibility [31,32]. In fact, the present results showed that both sealers had zero toxicity towards pre-osteoblastic cells, as supported by the similar cell count at each time point, relative the test group of cells placed in contact with the sealers and the respective control groups. This finding was confirmed by the absence of any significant differences in total protein levels between the test specimens and controls for NeoSealer Flo at each time point. With regard to AH Plus Bioceramic, slightly significant differences were detected between the test specimens and controls at each time point. However, these small differences were most likely not biologically relevant, given their variability and inconsistency and the generally positive trend, but were likely due to the small sample size and inherent methodological variables. Hydraulic calcium silicate-based sealers are characterized by an alkaline pH and a sustained release of calcium ions, features that have been shown not to adversely affect the homeostasis of surrounding tissues [33]. Rather, the pronounced release of calcium over time appears to be partly responsible for creating a pro-mineralizing environment that promotes osteogenic differentiation of pre-osteoblastic cells and subsequent mineral production [34,35]. Conversely, specific components leached from zinc oxide-eugenol-based and resin-based sealers have been reported to show marked cytotoxicity [15,31,36] or some evidence of moderate cytotoxicity [37]. These discrepancies may be related to the specific analytic methods applied. For example, some studies have allowed the sealers to fully set before testing their biocompatibility or have tested multiple increasing concentrations of various components derived from the sealers [31,37,38]. One aspect of the present study was facilitating direct semi-contact between the hydraulic calcium silicate-based sealer and cells, unlike many other studies in which the cells were placed in contact with sealer eluate, i.e., a medium previously incubated with the sealer sample [15,22,31,36]. This approach may have better reproduced the actual clinical condition. Interestingly, rapid development of crystals was observed when AHPB was placed in contact with the cells. This was not observed with Neo Sealer Flo. It can be suggested that these were calcium hydroxide precipitates released from the sealer itself reacting with the growth medium. Souza et al. reported a similar observation where a hydraulic calcium silicate-based sealer reacted with PBS [15]. However, the present data suggest that this had no significant effect on cell viability. To confirm this finding, longer contact times should be investigated. This different behavior might be explained by differences in the composition and physicochemical properties of the sealers. In fact, a recent study [11] comparing the present two sealers reported that Neosealer Flo had a significantly longer setting time and lower radiopacity than AH Plus Bioceramic. In addition, NeoSealer Flo showed higher solubility, although these differences were not significant. Both sealers maintained a similar alkaline pH from 3 h after complete setting and subsequently for over 28 days, while calcium release showed a cumulatively similar pattern, albeit with significantly higher initial values for NeoSealer Flo, but showing a progressive tendency for reversal of this trend. It should be noted, however, that the sealers were tested for pH and calcium release only after setting, and no data were obtained during the setting period. In addition, solubility, pH and calcium release were

tested by immersion in water, but not in other media such as PBS. Finally, NeoSealer flow showed significantly lower flowability and film thickness than AH Plus Bioceramic. Overall, however, both sealers met the specific ISO 6876/2012 standards for endodontic sealants [11].

In terms of protein expression, no obvious differences between the test specimens and controls for either of the hydraulic calcium silicate-based sealers were detected in the present study, as indicated by the SDS-PAGE assay. As characterization of the bioactivity was not the primary aim of this study, the method used for assessment of protein expression was merely able to provide a preliminary indication of any possible bioactive effects. However, a number of studies have already reported significant bioactivity in terms of osteogenic differentiation and tissue mineralization [31,35]. These preliminary analyses yielded promising results. Further studies should aim at investigating contact times longer than 72 h to detect any significant temporal changes in protein expression, including the use of complementary methods. Furthermore, the possible induction of pro-inflammatory molecules and mineralization ability should be evaluated to gain further insight into the interaction of different specific hydraulic calcium silicate-based sealers with the periodontal environment.

The present findings align partially with those of Sebastian et al. [36], who assessed NeoSealer Flo and TotalFill BC. Both studies confirmed that NeoSealer Flo had good biocompatibility, although the present results showed more stable viability over time. In terms of antibacterial activity, both studies reported moderate, time-sensitive effects against *E. faecalis*. Sebastian et al. observed decreased activity by day 7, consistent with the present observation of residual bacterial viability in the coronal third. Methodological differences, including the cell types and models used, may explain any minor discrepancies. Overall, both studies support the use of NeoSealer Flo as a biocompatible material with limited, time-dependent antimicrobial efficacy.

Furthermore, the antibacterial and cytocompatibility profiles of AH Plus Bioceramic observed in the present study are consistent with those reported by Chen et al. [39], who evaluated the properties of leachates from several endodontic sealers, including AH Plus Bioceramic. In both studies, the sealer exhibited limited antibacterial activity, with only moderate effects against *E. faecalis*, particularly in the early stages. This supports the hypothesis that the antimicrobial effect of calcium silicate-based sealers may be transient and time-sensitive. In terms of biological response, the high cell viability observed here was also in agreement with their observations [39]. However, Chen et al. [39] noted a temporary reduction in alkaline phosphatase (ALP) activity shortly after exposure, suggesting that ion release from the material might influence osteoblast function during the initial phase. While the present study did not evaluate ALP specifically, the absence of any cytotoxic effects and the stable protein expression are in line with a generally favorable biological profile. These comparisons reinforce the interpretation that although AH Plus Bioceramic maintains good biocompatibility, it may have limited and only short-term antibacterial potential, which seems relevant when considering its clinical application.

Clinically, these findings support the use of these sealers as biologically safe materials, particularly in cases requiring periapical healing. Nonetheless, the sealers should be considered as adjuncts and not substitutes for effective chemomechanical disinfection. Clinicians should be aware that although bioceramic sealers may promote biological compatibility and healing, their capacity to eliminate residual bacteria, especially in teeth with complex anatomy or in patients with persistent infections, may sometimes be insufficient. Therefore, the use of adjunctive strategies such as optimized irrigation protocols, activation techniques, and coronal sealing remains critical. Further *in vivo* and clinical studies are warranted for validation of these findings and assessment of long-term outcomes, particularly in relation to periapical healing, retreatment, and sealing performance over time.

In conclusion, exposure to the hydraulic calcium silicate-based sealers NeoSealer Flo and AH Plus Bioceramic Sealer resulted in no significant differences in bacterial viability. However, significant differences were observed in comparison to controls, and between the coronal and apical thirds of the tooth. The two sealers also showed good biocompatibility with pre-osteoblastic cells, suggesting that they can be used in close contact with periapical tissue without exerting any cytotoxic effects.

Abbreviations

α-MEM: alpha minimum essential medium; ANOVA: Analysis of variance; ATCC: American Type Culture Collection; BCA: bicinchoninic acid; BSA: bovine serum albumin; CFU: colony-forming units; CLSM: confocal laser scanning microscopy; DMSO: dimethyl sulfoxide; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; FBS: fetal bovine serum; MC3T3: murine calvarial pre-osteoblast cell line; NaOCl: sodium hypochlorite; *n*: number; O.D.: optical density; PBS: phosphate-buffered saline; PRILE: Preferred Reporting Items for Laboratory studies in Endodontology; rpm: revolutions per minute; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD: standard deviation; TSB: tryptic soy broth

Ethical Statements

This study was approved by the University Hospital of Siena Ethics Committee (Siena, Italy), Area Vasta Toscana Sud Est, protocol number Protocol (no.7/2021). All enrolled patients were informed about the study protocol and were asked to read and sign an informed consent form. The present study was conducted according to the Declaration of Helsinki.

Conflicts of Interest

The authors have no conflicts of interest to report in relation to this study.

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Author Contributions

CG: supervision, resources, writing – review & editing. FV: formal analysis, validation, methodology, writing – review & editing. GM: conceptualization, methodology, data curation, investigation, writing – original draft, writing – review & editing, visualization. JB: investigation, methodology. FC: validation, supervision. OS: investigation, data curation. ET: investigation. CF: project administration, resources. SG: conceptualization, supervision, funding acquisition. LG: methodology, writing – review & editing, supervision. All authors approved the published version of the manuscript.

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Data Availability Statements

The data sets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

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