Original Article

Evaluation of the antibacterial and cytotoxic properties of TotalFill and NeoSEALER flo bioceramic sealers

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Abstract

Aim: Evaluation of the antibacterial and cytotoxic properties of TotalFill and NeoSEALER Flo bioceramic sealers compared to AH Plus resin sealer.

Materials and Methods: Modified direct contact test was used on three sets of sealers: Freshly mixed sealers, sealers that were 1-day old, and sealers that were 7-day old. After 24 h of incubation, the colony-forming units were digitally counted using Promega Colony Counter after 30 and 60 min of exposure to *Enterococcus faecalis*. For cytotoxic effect evaluation, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay was performed at three different time points: 24 h, 48 h, and 120 h after adding the sealer eluates to human gingival fibroblasts, to assess cell viability. Data were analyzed using mixed model analysis of variance followed by *post hoc* test.

Results: TotalFill bioceramic sealer showed the highest bacterial reduction against *E. faecalis* throughout all intervals. AH Plus showed great antibacterial activity initially which reduced drastically after 7 days. All the sealers showed a reduction in their antibacterial activity with time. TotalFill and NeoSEALER Flo showed very high cell viability in contrast to AH Plus.

Conclusion: TotalFill and NeoSEALER Flo demonstrate superior antimicrobial properties against *E. faecalis* which reduces with time. TotalFill and NeoSEALER Flo demonstrate acceptable biocompatibility against human gingival fibroblasts, which decreased over time.

Keywords: 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay; bioceramics; *Enterococcus faecalis*; human gingival fibroblasts; modified direct contact test

INTRODUCTION

Endodontic treatment aims to achieve a thoroughly cleaned and properly shaped root canal system by employing effective instrumentation techniques and suitable irrigation solutions. This process is followed by a three-dimensional sealing step, which further reduces or eliminates microorganisms within the dental root canal.[1] The endodontic sealers used in the obturation process of root canals must possess specific qualities such as appropriate marginal properties, low solubility, and an adequate setting time. They should also be impermeable, exhibit antibacterial properties, be biocompatible, and be nontoxic.[2]

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From a clinical perspective, most root canal sealers are designed to be introduced into the root canals while in a fresh state with a flowable consistency. However, this approach carries the risk of the sealer being extruded beyond the apical constriction, escaping through the apical foramen, and entering the periapical tissues. Such contact between the sealer and periapical tissues is undesirable as it can lead to various tissue reactions, including tissue degeneration and delayed wound healing, depending on the composition of the extruded sealer.[3]

Bioceramic sealers offer improved physicochemical and biological properties, providing an alternative to overcome the drawbacks associated with epoxy resin-based sealers.[4-6] An example of such a sealer, TotalFill BC Sealer, manufactured by FKG, is part of the most recent iteration within this sealer category. This product is an injectable bioceramic sealer, preprepared for use, and it comprises components such as calcium silicates, calcium hydroxide, calcium phosphate, thickening agents, and zirconium oxide, which serves as a radiopacifier. This sealer is known for its biocompatibility and antimicrobial activity. The presence of water is mandatory to achieve a complete setting.[7,8] In addition, due to its high pH and ability to release calcium hydroxide, this sealer has the potential to exhibit an antimicrobial effect against *Enterococcus faecalis*.[9-11]

NeoSEALER Flo, manufactured by Avalon BioMed, is an innovative bioactive bioceramic sealer. As stated by the manufacturer, this sealer is comprised of a tricalcium and dicalcium silicate powder of a very small particle size. These inorganic components are suspended in an organic medium, creating a unique formulation for the sealer. The manufacturer highlights its exceptional handling properties, which contribute to its ease of use during root canal procedures. Moreover, NeoSEALER Flo is specifically formulated to stimulate the formation of hydroxyapatite, an essential component for facilitating the healing process in the treated area.[12]

AH Plus is an epoxy resin-based root canal sealer that possesses several advantageous properties. It has been extensively tested demonstrating low microleakage, the capability to bond to dentin, antibacterial activity against *E. faecalis*, and dimensional stability due to minimal polymerization shrinkage upon entering the root canal.[10,13,14]

The biological properties of these novel bioceramic sealers have not been fully tested. Therefore, the current study aims to evaluate the antibacterial properties and cytotoxic effects of TotalFill and NeoSEALER Flo bioceramic root canal sealers in comparison to AH Plus resin sealer using modified direct contact test (mDCT) and 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay, respectively. The null hypothesis tested is that there is no statistically significant difference between the antibacterial properties and cytotoxic effect of TotalFill and NeoSEALER Flo compared to AH Plus resin sealer.

MATERIALS AND METHODS

Samples preparation

For the antibacterial study, the sealers were mixed and used for the fresh group placed at the bottom of a vertically held 12-well plate, and stored in the incubator to set for 1 day and 7 days. For the cytotoxic study, discs of the sealers were prepared in polyvinyl chloride ring molds, and the respective eluates were collected and stored for the experiment.

Antimicrobial evaluation

A few colonies of *E. faecalis* were cultivated on a Brain Heart Infusion (BHI) agar plate. The plate was then incubated at 37°C for 24 h. A small number of *E. Faecalis* (ATCC strain 29212) colonies were cultured in 1 mL of BHI broth to verify their purity. The culture was then incubated at 37°C for 24 h. Following this, the inoculum with *E. faecalis* was adjusted to attain the turbidity equivalent to 0.5 McFarland Standard (approximately 1.5×10^8 colony-forming units [CFUs]/mL).

Modified direct contact test

The mDCT was employed to assess the antibacterial properties of the sealers. Within vertically held 12-well cell culture plates, the sealers were applied. A precise measurement of 20 mg for each sealer was obtained using a digital laboratory weighing balance accurate to 0.01 g/0.001 g, and this amount was placed at the bottom of each well. The samples tested immediately after mixing were categorized as the "fresh group." Samples subjected to 1-day and 7-day setting periods in a humid environment at 37°C before testing were labeled as the "1-day aged group" and "7-day aged group," respectively. In the control groups, the positive controls consisted of wells without sealer coating, into which 20 µL of bacterial suspension was added. Furthermore, for the negative control, sealers without the addition of bacterial suspension were utilized.[15]

Within each sealer group, 20 µL of a bacterial suspension was dispensed into the wells, followed by a 30-and 60-min incubation period. Using a micropipette, 180 µL of sterile saline was introduced into each well. The suspension was gently mixed, and the entire microbial suspension from each well was subsequently transferred and subjected to serial dilution in sterile saline. From each serially diluted test tube in both the test and control groups, 20 µL portions were extracted and cultured on BHI agar plates with appropriate labeling. These plates were then

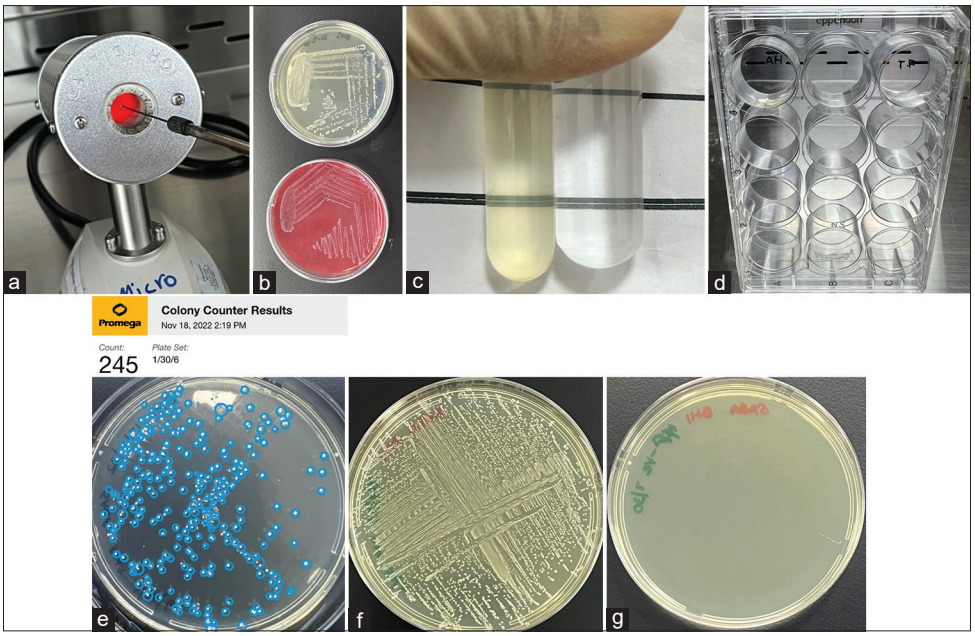

Figure 1: Antibacterial testing. (a) Heat sterilization of the metal loop. (b) Subculture of *Enterococcus faecalis* from blood agar. (c) Turbidity equivalent to 0.5 McFarland standard turbidity. (d) Vertically held 12 well culture plate with the sealers at the bottom of the well. (e) colony-forming unit count for NeoSEALER Flo. (f) Culture of positive control showing maximum growth of *E. faecalis*. (g) Culture of negative control showing no growth of *E. faecalis*

incubated for 24 h at 37°C, after which the colonies were digitally counted, as illustrated in Figure 1. This process was conducted in triplicate.[15]

The logarithmic value of the CFUs for all samples was determined. The assessment of bacterial reduction was performed based on the following calculations.

Bacterial reduction = Positive control – Log CFU.

Cytotoxicity evaluation

The effect of sealers was examined on human gingival fibroblasts using their eluates. Three discs of each sealer were prepared and were exposed to ultraviolet rays on both sides. After sterilization, 3 mL of Dulbecco's Modified Eagle Medium (DMEM) was added to each sealer disc in individual Eppendorf Tubes and incubated for 24 h. This medium, which contained the sample, was referred to as the test medium.[16]

3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay

The cells were counted and then plated in 96-well plates for the assay at different time points. Specifically, 320,000 cells per 200 µL were plated for the 24-h assay, 80,000 cells per 200 µL were plated for the 48-h assay, and 20,000 cells per 200 µL were plated for the 120-h assay. All the plates, including those for the control and each sealer, were placed in a humid incubator with 5% CO_2 and 95% air for 24 h. The negative control consisted of cells in DMEM growth media, whereas the positive control had DMEM alone. The medium was removed from the 96-well plates after 24 h, and then the test medium was added to all wells except for the controls and the plates were incubated for an additional 24 h. MTT assay was conducted to analyze the cell viability with the absorbance of each well in the 96-well dish measured using an automatic microplate spectrophotometer at 570 nm. For the cells in which the MTT assay was scheduled to take place after 120 h, the test media and growth media for the controls were renewed after the initial 72 h. The experiment was conducted in triplicates at 24, 48, and 120 h, and the process was repeated three times as shown in Figure 2.[16]

Statistical analysis

Data showed parametric distribution using the Shapiro–Wilk test and were analyzed using mixed model analysis of variance followed by comparisons of estimated marginal means using multiple *t*-tests with *P* value adjustment using Tukey's method. The significance level was set at $P < 0.05$. Statistical analysis was performed with R statistical analysis software version 4.3.0 for Windows (The R Foundation, Auckland, New Zealand).

RESULTS

Modified direct contact test

Sealer effect

Mean and standard deviation values of log bacterial reduction for different sealers are presented in Table 1.

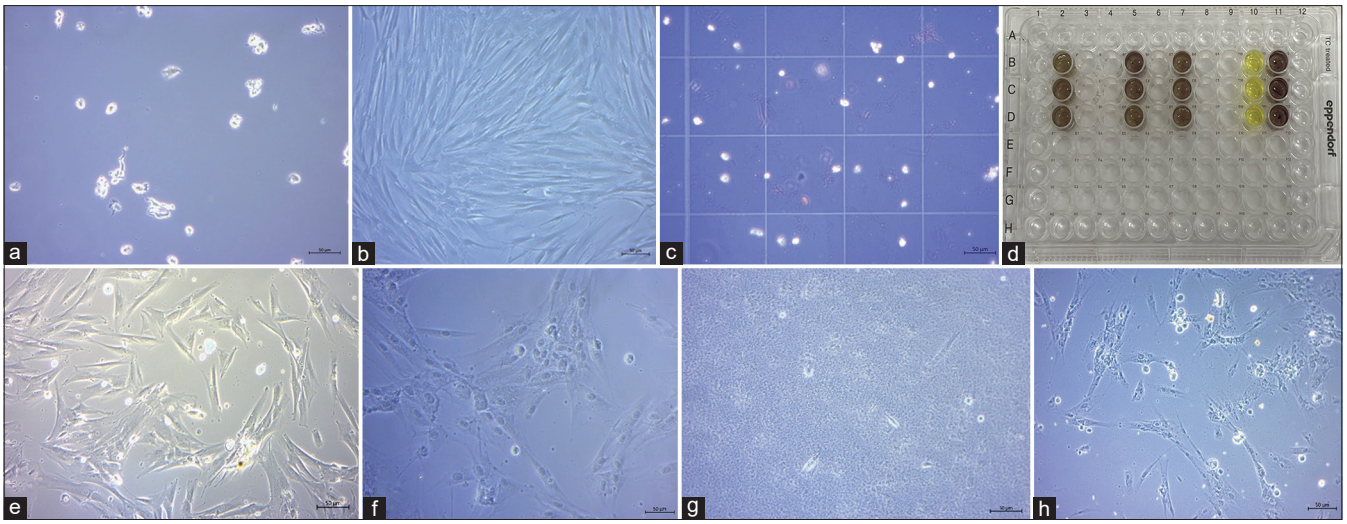

Figure 2: Cytotoxicity testing. (a) Floating cells. (b) Cell confluency. (c) Fibroblast cells inside the counting grid of the hemocytometer. (d) A 96-well-plate after 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay, column 2, 5, 7, 10, and 11 has AH plus sealer, NeoSEALER Flo, Totalfill sealer, positive control and negative control, respectively. (e) Cell morphology of the negative control. (f) Cell morphology after treatment with AH plus resin sealer. (g) Cell morphology after treatment with NeoSEALER flo. (h) Cell morphology after treatment with TotalFill BC sealer

Table 1: Mean and standard deviation values of log bacterial reduction for different sealers

State	Time (min)	Bacterial reduction log (mean±SD)				*P*
		AH plus	NeoSEALER Flo	Totalfill sealer	Control	
Freshly mixed	30	6.66 ± 0.01^{A}	6.32 ± 0.37^{B}	6.66 ± 0.01^{A}	6.66 ± 0.00^{A}	<0.001*
	60	6.73 ± 0.00^{A}	6.29 ± 0.28^{B}	6.71 ± 0.03^{A}	6.73 ± 0.00^{A}	<0.001*
1-day aged	30	6.31 ± 0.27^{B}	6.16 ± 0.35^{B}	6.19 ± 0.29^{B}	6.66 ± 0.00^{A}	<0.001*
	60	6.48 ± 0.21^{A}	6.70 ± 0.04^{A}	6.11 ± 0.41^{B}	6.73 ± 0.00^{A}	<0.001*
7-days aged	30	6.38 ± 0.20^{A}	5.87 ± 0.78^{B}	6.46 ± 0.15^{A}	6.66 ± 0.00^{A}	0.001*
	60	6.14 ± 0.35^{C}	$6.38\pm0.22^{B,C}$	$6.62\pm0.09^{A,B}$	6.73 ± 0.00^{A}	<0.001*

*Significant ($P\leq0.05$). Different superscript letters indicate a statistically significant difference within the same horizontal row. NS: Nonsignificant ($P>0.05$), SD: Standard deviation

Bacterial contamination time effect

Mean and standard deviation values of bacterial reduction log count (CFU) for different times after introducing bacteria to different sealers are presented in Table 1.

3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay

Sealer effect

The mean and standard deviation values of cell viability (%) for different sealers are presented in Table 2.

Time effect

Mean and standard deviation values of cell viability (%) for different times are presented in Table 3.

DISCUSSION

Endodontic sealers serve a crucial purpose in root canal therapy by effectively eliminating microorganisms that persist after the chemomechanical preparation and by preventing the reestablishment of these microorganisms within the intricate root canal system. An endodontic sealer should exhibit a sustained and durable antibacterial effect.[17] In this context, *E. faecalis*, a Gram-positive bacterium known for its ability to endure high pH conditions and nutritional deprivation, has been commonly employed in studies to evaluate the antibacterial effectiveness of different sealers.[18]

The antibacterial experiment used in this study was the mDCT method. This is a reliable and reproducible method used to quantitatively assess the antibacterial effects of insoluble materials, specifically in evaluating the antimicrobial properties of root canal sealers. This technique involves introducing the bacteria to the test materials, serially diluting the concentrations, and digitally counting the number of microbial colonies, providing a quantitative assay.[15] The experiment in the study was performed in triplicates to improve the accuracy and reproducibility of the results.

Biocompatibility is another vital characteristic that an ideal sealer should possess. When sealers or their components come into contact with the periapical tissues, they can elicit a local inflammatory response. This inflammatory reaction has the potential to compromise the success of the root

Table 2: Intergroup comparisons, mean and standard deviation values of cell viability (%) for different sealers

Time (h)	Cell viability (%) (mean±SD)					*P*
	AH plus	NeoSEALER Flo	Totalfill sealer	Positive control	Negative control	
24	16.93±2.06^{C}	71.49±4.46^{B}	69.56±0.74^{B}	4.72±0.19^{D}	100.00±0.00^{A}	<0.001*
48	0.59±0.37^{E}	51.82±1.14^{C}	70.28±1.29^{B}	5.74±0.46^{D}	100.00±0.00^{A}	<0.001*
120	1.09±0.56^{D}	62.54±5.57^{B}	53.37±3.17^{C}	5.97±0.88^{D}	100.00±0.00^{A}	<0.001*

*Significant ($P \leq 0.05$). Different superscript letters indicate a statistically significant difference within the same horizontal row. NS: Nonsignificant ($P > 0.05$), SD: Standard deviation

Table 3: Intergroup comparisons, mean and standard deviation values of cell viability (%) for different times

Sealer	Cell viability (%), mean±SD			*P*
	24 h	48 h	120 h	
AH plus	16.93±2.06^{A}	0.59±0.37^{B}	1.09±0.56^{B}	<0.001*
NeoSEALER Flo	71.49±4.46^{A}	51.82±1.14^{B}	62.54±5.57^{C}	0.011*
Totalfill sealer	69.56±0.74^{A}	70.28±1.29^{A}	53.37±3.17^{B}	<0.001*
Positive control	4.72±0.19^{A}	5.74±0.46^{A}	5.97±0.88^{A}	0.106 NS
Negative control	100.00±0.00	100.00±0.00	100.00±0.00	NA

*Significant ($P \leq 0.05$). Different superscript letters indicate a statistically significant difference within the same horizontal row. NS: Nonsignificant ($P > 0.05$), NA: Not available, SD: Standard deviation

canal treatment, even if the root canal debridement and disinfection were appropriately performed.[19]

Several types of assays can be used to determine the number of viable cells. The technique employed for evaluating the cytotoxic impact in this study is the MTT assay. This is a commonly utilized colorimetric approach for appraising cytotoxicity or cell viability. It assesses cell viability by gauging the activity of mitochondrial enzymes, with a particular focus on succinate dehydrogenase, which serves as an indicator of mitochondrial function.[9]

In the present study, the different test groups of the antibacterial study and the cytotoxic study showed significant differences in their antibacterial effects and cytotoxicity, thereby rejecting the null hypothesis.

TotalFill BC sealer has shown the highest antibacterial properties at all time intervals. It has an active calcium hydroxide diffusion leading to an increase in pH, which might have resulted in an effective antibacterial property. The elevated pH creates an unfavorable environment for bacterial growth as the bacteria usually sustain in an acidic environment, whereas the diffusion of calcium hydroxide further enhances its antibacterial properties. This is following a study conducted by Janini *et al*., who found that TotalFill BC sealer has a superior antibacterial effect compared to other sealers after 24 h.[20]

The significant bactericidal impact of AH Plus sealer could be linked to the emission of formaldehyde as a byproduct during the polymerization process, which is confirmed by Janini *et al*. who found that AH Plus showed a significant antimicrobial effect which includes bactericidal effect in the first 24 h.[20]

There was a reduction in the antibacterial potential of AH Plus compared to the bioceramic sealers by 7 days of setting. The antimicrobial activity of AH Plus is primarily observed in the short term, which diminished significantly with time. It could be potentially due to the toxicity of certain unpolymerized components such as unset epoxy resin and amines.[21] The notable reduction in the antibacterial efficacy of the AH Plus may be ascribed to the polymerization process, which leads to a depletion of the epoxy resin and amines.[15]

Bioceramic sealers showed a decrease in the bacterial reduction in 1-day aged sealer which again finds an increase in the 7-day aged sealer group. The effectiveness of calcium hydroxide-based materials in killing bacteria relies on the release of hydroxyl ions, which leads to a rise in pH. The pH increase can either reversibly or irreversibly deactivate cellular membrane enzymes in microorganisms. This comes in full agreement with Koruyucu *et al*. who found similar findings of increased antibacterial activity of bioceramic sealers in freshly mixed and 1-week samples.[22]

TotalFill continued to show the highest antibacterial effect even in the 7-day-aged sealer group. This indicates that TotalFill BC Sealer has a more pronounced ability to inhibit the growth of *E. faecalis* compared to AH Plus.[19] This superior activity can likely be attributed to the presence of monocalcium phosphate, a compound that is unique to TotalFill BC Sealer and contributes to its enhanced antimicrobial efficacy.[20] Some of the chemical compounds with alkaline properties found in the composition of bioceramic endodontic sealers might contribute to their antimicrobial effects.[9,19]

All the sealers showed a better antibacterial effect after 60 min of bacterial contamination time than that of 30 min. This might be because of the increased duration of the effect of the calcium hydroxide diffusion and high pH on the bacteria which has resulted in more bacterial reduction after 60 min. Alternatively, the sealers do not have enough time to exert their antibacterial influence against *E. faecalis*. Poggio *et al*., in a similar study, to evaluate the antibacterial activity through direct contact test, suggested a time of 60 min of bacterial contact than 6 and 15 min, citing the same reason.[23]

Although NeoSEALER Flo and TotalFill sealer are bioceramic sealers, NeoSEALER Flo has presented with significantly

lesser antibacterial activity than TotalFill sealer. This difference in the bacterial reduction could be attributed to the lack of the chemical compound, monobasic calcium phosphate, which is present in TotalFill sealer. In a study by Zamparini *et al*., NeoSEALER Flo was found to have lower alkalinizing activity and lower calcium release,[12] which might contribute to the lower antibacterial effect presented. To the best of our knowledge, no previous study has evaluated the antibacterial effect of NeoSEALER Flo bioceramic sealer on any microorganisms.

AH Plus exhibited notably reduced cell viability after 24 h. Throughout all the time intervals, AH Plus demonstrated the highest levels of cytotoxicity, which may be attributed to the genotoxic consequences of formaldehyde.[20] Following 48 and 120 h, AH Plus left almost no viable cells, and it was consistently observed to exhibit cytotoxicity in various cell line experiments. Mak *et al*. suggested that the reduced cell viability may be linked to both the epoxy resin and the amines.[24] Epoxy resin-containing sealers have been evaluated for their cytotoxic properties, primarily because of their component bisphenol (diglycidyl ether), which is recognized for its mutagenic characteristics.[10] Among all the sealers tested, AH Plus consistently exhibited the least cell viability throughout all the experimental time intervals.

In this study, the calcium silicate-based sealers displayed superior cell viability when compared to AH Plus. The improved cell viability percentages observed with calcium silicate-based bioceramic sealers, such as TotalFill, can be attributed to increased calcium ion release, a basic pH environment, and the formation of hydroxyapatite. Furthermore, the capacity for releasing calcium ions plays a crucial role in promoting periapical tissue repair. The interfacial apatite layer bonds the bioceramic sealer and the radicular dentin, potentially leading to enhanced cell viability.[10]

NeoSEALER Flo presented the highest cell viability, significantly higher than AH Plus, respectively, at each evaluation time interval, which could be attributed to the osteoconductive manner and high alkalinity which provides a continued release of calcium ions. The findings in this study are in line with that of Elgendy and Badr 2023.[25]

All the sealers showed a decrease in cell viability with time. Due to the AH Plus setting through a polymerization system known as "Linear Epoxide-Amine Addition," the gradual release of unreacted monomers from its polymer matrix could explain its cytotoxicity to increase over an extended time.[26]

The growing cytotoxic impact of bioceramic sealers over time could be attributed to their elevated pH levels.[27] Calcium silicate-based sealers exhibit the highest levels of Ca^{+2} release and alkalizing activity, which can be ascribed to variations in the proportions of calcium silicates and calcium aluminates.[12] The high calcium hydroxide release and alkaline pH of these sealers can be sufficiently irritating to cause severe inflammatory responses, which cause denaturation of adjacent cells, thereby proving to be toxic.[10] These results are in agreement with the studies of Lee *et al*. and Jagtap *et al*. which found a decrease in cell viability of the bioceramic sealers with time.[27,28]

Although the results of this study open the door to building scientific evidence for the bioceramic sealers, many more investigations looking into biocompatibility against different stem cells are required. Further studies on the physical and biological properties of novel sealers like NeoSEALER Flo are necessary to understand the chemical and mechanical properties of the materials.

CONCLUSION

TotalFill demonstrates superior antimicrobial properties against *E. faecalis* consistently across all time intervals. In general, aged bioceramic sealers require adequate time to exhibit their antimicrobial properties effectively. The antibacterial activity decreases over time. Bioceramic sealers tested demonstrate acceptable biocompatibility against human gingival fibroblasts, although this biocompatibility decreases over time.

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Conflicts of interest
There are no conflicts of interest.

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