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RESEARCH PAPER

The antibacterial activity of an epoxy resin-based dental sealer containing bioactive glass, hydroxyapatite, and fluorohydroxyapatite nanoparticles against *Enterococcus Faecalis* and *Streptococcus* mitis

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ABSTRACT

Objective(s): The present study aimed to investigate the antibacterial properties of a conventional epoxy-based dental sealer modified with synthesized bioactive glass (BG), hydroxyapatite (HA), and fluorine-substituted hydroxyapatite (FHA) nano-fillers.

Materials and Methods: The synthesized nano-fillers were incorporated into the conventional epoxy-based dental seaer at the concentration of 10%. The antimicrobial properties of the unmodified sealers (controls) and modified sealers with BG, HA, and FHA nanoparticles (NPs) were evaluated based on biofilm formation and using the direct contact test (DCT) of Enterococcus faecalis and Streptococcus mitis. Data analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post-hoc test at the significance level of 5%.

Results: A significant reduction was observed in the biofilm formation and DCT of the microbial strains in the three modified groups compared to the unmodified conventional epoxy sealer ($P<0.05$). The addition of FHA NPs resulted in the most significant antibacterial effects against *E. faecalis* and *S. mitis*, as well as a statistically significant reduction compared to the unmodified and BG-modified groups ($P\leq0.001$).

Conclusion: According to the results of this preliminary study, nano-structured FHA, HA, and BG fillers incorporated into epoxy-based dental sealers could be potentially effective biomaterials for antibacterial approaches to root canal treatments.

Keywords: *Bioactive Glass, Enterococcus faecalis, Fluorine-substituted Hydroxyapatite, Hydroxyapatite, Nanoparticles, Streptococcus mitis*

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INTRODUCTION

Successful endodontic treatment relies on the complete elimination of bacterial infections and sealing of the root canal system [1]. However, the instrumentation, irrigation, and inter-canal medications could significantly reduce the bacterial population of an infected root canal system, thereby making the complete elimination of all microorganisms virtually impossible [2]. Therefore, root canal filling materials such as obturation materials and sealers with antibacterial properties are commonly applied in order to further reduce the concentration of the remaining

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microorganisms [2].

Various bacteria are detected in the primarily- or secondarily-infected root canal systems. *Enterococcus faecalis* and *Streptococcus* spp. are often detected in persistent and secondary dental root canal infections. Most *Streptococcus* species consists of *S. anginosus* and *S. mitis* [3, 4]. *E. faecalis* is reported to be the most prevalent bacterial strain in infected root canals, as well as re-treatment cases. *E. faecalis* may deeply invade the dentinal tubules and survive during routine root canal treatments; as such, its eradication is rather complicated [5, 6].

Since the sealer inside the root canal system may be in direct contact with the remaining microorganisms, various studies have experimented with the incorporation of antibacterial materials into root canal sealers [7]. Reports have confirmed the effective antibacterial activity of benzalkonium chloride, cetylpyridinium chloride [7] amoxicillin [8-10], chlorhexidine with a-tricalcium phosphate [11], and quaternary ammonium polyethylenimine (QPEI) [12] while successfully incorporated into various root canal sealers.

In the past few decades, nanoparticles (NPs) with dimensions of 1-1,000 nanometers have been studied owing to their unique physicochemical properties. Particles with nanometer-scale dimensions have higher surface areas, which is associated with their effective interaction with microorganisms [13]. Furthermore, various nano-materials have been used to enhance the antibacterial activity of root canal sealers and filling materials, such as chitosan NPs, bioactive glass (BG) NPs, silver NPs, QPEI NPs, and zinc oxide NPs [14].

BGs are mainly composed of variable concentrations of SiO_2, CaO, Na_2O, and P_2O_5 and are extensively applied in dentistry considering their ability to induce apatite formation [15, 16], as well as their remarkable antibacterial properties [13, 14]. Some of the main influential factors in the antibacterial activity of BG NPs are increased pH due to ion release, increased osmotic pressure (>1%), and the ability to induce mineralization on the surface of bacteria [14].

Hydroxyapatite [HA; $Ca_5(PO_4)_3OH$] has been widely used in dentistry owing to its high biocompatibility, similarity to human hard tissues, bioactivity [17, 18], and antibacterial properties [19, 20]. The complete and partial substitution of a fluoride ion (F^-) with a hydroxyl ion (OH^-) results in the generation of fluorapatite [FA; $Ca_5(PO_4)_3F$] and fluoride-substituted hydroxyapatite [FHA; $Ca_5(PO_4)_3(OH)_{1-x}F_x$], respectively [21]. The substitution resulted in the reduction of the unit cell volume and enhancement of the density and chemical stability of the apatite structure [22]. On the other hand, the effects of FHA and FA on oral bacteria and dental plaque were attributed to the release of fluorine ions, which could variably influence microorganisms as documented in the current literature [23-25].

The present study aimed to investigate the antibacterial activity of epoxy-based endodontic sealers containing BG, HA, and FHA NPs on *E. faecalis* and *S. mitis.* The null hypothesis was that the addition of nanostructured BG, HA or FHA to the epoxy-based endodontic sealers has no effects on antibacterial properties and biofilm formation.

MATERIALS AND METHODS

Sample preparation for study groups

In this study, silver-free, commercially available AH26 epoxy resin sealer (Dentsply Maillefer, USA) was modified using BG, HA, and FHA NPs, which were synthesized and characterized as previously described [26].

For the synthesis of HA NPs, diammonium hydrogen phosphate solution (0.15 M; Merck, Germany) was added to calcium nitrate solution (0.09 M) with vigorous stirring at the pH of 11. The resultant precipitate powder was aged at room temperature for 24 hours. Following that, the precipitated powder was centrifuged, washed with deionized water, and dried. The FHA powder was synthesized with the addition of the prepared HA powder (0.02 M) to sodium fluoride solution (0.02 M, 98.5%; Merck, Germany) at the pH of seven. Afterwards, nitric acid (1 M, 68%; Merck, Germany) was added, and the pH decreased to four. The pH reached seven following the addition of sodium hydroxide (1 M). The pH cycle was repeated three times, and the resultant solution was centrifuged and washed with deionized water, and the obtained wet powders were dried.

The BG NPs were synthesized with the addition of tetraethyl orthosilicate (5.8 mol) to the nitric acid solution (1 M) and mixed for one hour. Afterwards, 5.8 mol of triethyl phosphate, 0.17 mol of calcium nitrate tetrahydrate, and 0.32 mol of sodium nitrate ($NaNO_3$) were added and allowed to react for one hour. The resultant transparent

solution was stored for five days at room temperature, after which gel formation occurred. The prepared gel was aged for 24 hours, dried at the temperature of 120°C, and calcined at the temperature of 700°C. The synthesized powders were ball-milled at 1,200 rpm for 10 minutes and characterized via XRD, field emission scanning electron microscopy micrographs (FE-SEM), and Fourier-transform infrared spectroscopy (FTIR). The details of the characterization process are described in our unpublished study.

The microstructure of the HA powders was almost round-shaped, and the particle sizes were within the range of 20-80 nanometers in diameter. The BG particles had irregular shapes, with their particle sizes within the range of 30-60 nanometers. The morphology of the FHA particles was almost plate-like, and their width ranged from 200 nanometers to one micrometer (Fig 1).

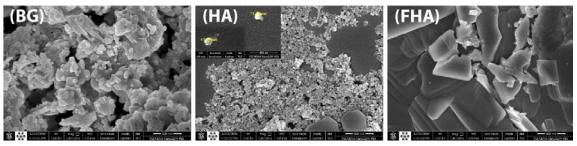

Fig 1. Field emission scanning electron microscopy (FESEM) micrographs of synthesized BG, HA, and FHA powders

The synthesized nano-powders and powder of the conventional epoxy-based dental sealer (70% bismuth oxide and 25% hexamethylenetetramine) were weighed using an analytical balance (Sartorius, Germany) and divided into equal parts. Each part of the synthesized nano-powders was manually mixed with the powder of the commercially available dental sealer. Following that, the powders were mixed using a dental amalgamator device (Ultramat 2, SDI, Australia) in order to achieve appropriate particle distribution.

At the next stage, the unmodified powder of the epoxy sealer and sealers modified with 10% synthesized nano-fillers were mixed with a commercially available epoxy resin liquid (bisphenol A-diglycidyl ether) with the powder-to-liquid ratio of 2:1 in accordance with the instructions of the manufacturer. The cylindrical specimens (n=5) with specified dimensions (diameter: 6 mm, height: 2 mm) were prepared for each experimental group. In order to ensure complete polymerization, all the prepared specimens were incubated at the temperature of 37°C and 100% humidity. The fabricated samples were sterilized using gamma rays (25 kGy). Afterwards, the prepared specimens were divided into four groups based on the type of the added nano-fillers. Correspondingly, the study groups included control, BG NPs, HA NPs, and FHA NPs.

Bacterial strains and growth conditions

In the current research, *E. faecalis* ATCC 29212 and *S. mitis* ATCC 6249 were obtained from the Iranian Biological Resource Center (Tehran, Iran). *E. faecalis* and *S. mitis* were grown in aerobic and microaerophilic conditions, respectively on brain heart infusion (BHI) broth (Merck KGaA, Germany) at the temperature of 37°C until the cells attained the concentration of 1.0×10^8 colony-forming units (CFU)/ml^{-1} as verified by the optical density of 600 nanometers within the range of 0.08-0.13 using a spectrophotometer (Eppendorf BioSpectrometer® Fluorescence, Germany).

Biofilm formation

The biofilm formation of each bacterial strain was evaluated independently on the surface of the prepared samples that were placed in the tubes containing suspensions with the mentioned bacteria. In total, 1.5×10^8 CFU/ml of each strain was grown in trypticase soy broth (Merck, Germany) containing 0.5% glucose and incubated at the temperature of 37°C for 48 hours. After incubation, the samples were gently washed with three milliliters of sterile phosphate-buffered saline (10 mM of Na_2HPO_4, 2 mM of NaH_2PO_4, 2.7 mM of KCl, 137 mM of NaCl, and pH of 7.4) in order to remove the loose, non-adherent bound cells. Two samples were processed for scanning electron microscopy (SEM) so as to confirm the biofilm formation of *E. faecalis* and *S. mitis* on the surface of the samples.At the next stage, the specimens were placed in the tubes containing one milliliter of the BHI broth and sonicated using a high-speed ultrasonic homogenizer (Sonoplus UW2200, Bandelin, Germany) at 100 W and frequency of 30 kHz for 30 seconds. The obtained microbial suspensions were serially diluted, spread-cultured on BHI agar (Merck KGaA, Germany) using sterile spreaders, and incubated at the temperature of 37°C for 24 hours.After incubation, the viable bacteria were counted, and the CFU/ml^{-1} values were determined using the method proposed by Miles and Misra [27]. The samples that were exposed to 2.5% NaOCl and contaminated with the bacterial strains only were considered as the negative and positive controls, respectively.

Direct contact test (DCT)

In the DCT, the prepared samples were placed in the tubes containing one milliliter of the

microbial suspensions with the final concentration of 1.5×10^5 CFU/ml^{-1}. The tubes were incubated in a shaking incubator at 120 rpm for 24 hours at the temperature of 37°C based on the growth conditions of each microorganism. Following that, the obtained suspensions were serially diluted and spread-cultured on the BHI agar. The microbial colony counts were determined as described in the previous section.

Statistical analysis

Data analysis was performed in SPSS version 23.0. The normality and homogeneity of the data variances were assessed and confirmed using the Kolmogorov-Smirnov test and Levene's test, respectively at the significance level of 5%. In addition, one-way analysis of variance (ANOVA) and Tukey's post-hoc test were used, with the P-value of less than 0.05 considered statistically significant.

RESULTS

Biofilm formation

In the present study, we evaluated the formation of a mature biofilm on the surface of the conventional epoxy-based sealer and modified sealers with three calcium phosphate synthetic NPs. The SEM microphotograph of the randomly assigned samples confirmed the presence of *E. faecalis* and *S. mitis* biofilms after inoculation. The counts of the viable bacteria in the study groups are presented in Table 1.

Table 1. Viable counts (mean ± SD) of *E. faecalis* and *S. mitis* in un-modified conventional dental sealer (control group) and those modified with BG, HA and FHA NPs

	Microbial strains (CFU mL^{-1}) (mean LogCount ± SD)			
Groups (n=5)	Control	BG	HA	FHA
E. faecalis	6.32±0.05^{abc}	6.21±0.06^{ade}	5.40±0.04^{bd}	5.35±0.03^{ce}
S. mitis	6.18±0.05^{fgh}	5.94±0.1^{fij}	5.27±0.05^{gi}	5.14±0.09^{hj}

The results of one-way ANOVA indicated a significant reduction in the biofilm formation of the two microbial strains on the surface of all the modified groups compared to the unmodified conventional epoxy sealer (P<0.05). In this regard, the significance level obtained by Tukey's post-hoc test between the control group and BG-modified samples for *E. faecalis* and *S. mitis* was estimated at 0.01 and 0.02, respectively, while the significance level between the control group and two other groups for both microbial strains was determined to be ≤0.001. However, the difference in the counts of the viable bacteria between the HA- and FHA-modified samples of *E. faecalis* and *S. mitis* was not considered statistically significant (P=0.13 and P=0.39, respectively) (Fig 2).Fig 3 depicts the representative images of the viable microbial counts in terms of biofilm formation on the surface of the prepared experimental samples.

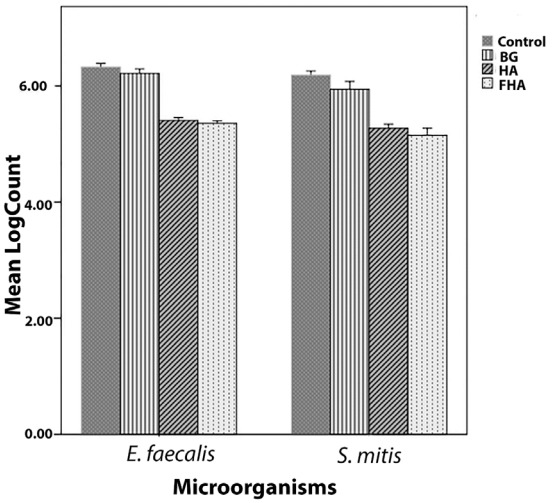

Fig 2. Viable Microorganism Counts (CFU/ml-1) of *E. faecalis* and *S. mitis* in Biofilm of Study Groups (unmodified control and specimens modified with BG, HA, and FHA NPs)

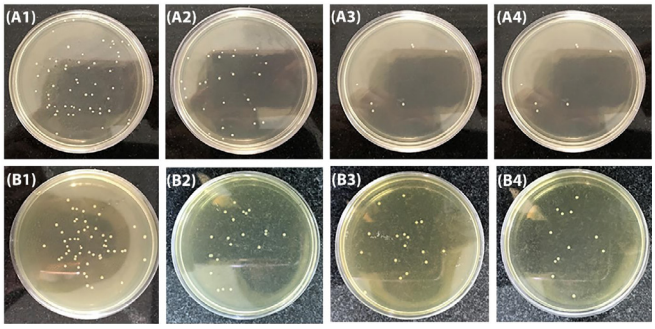

Fig 3. Image of Microbial Count of A) *E. faecalis* and B) *S. mitis* in Biofilm Formed in A1, B1) Unmodified Specimens (control) and A2, B2) Specimens Modified with BG NPs, A3, B3), HA NPs, and A4, B4) FHA NPs

The same letters denote the groups which have statistically significant differences.

Table 2. Viable counts (mean ± SD) of *E. faecalis* and *S. mitis* obtained by DCT in un-modified conventional dental sealer (control group) and those modified with BG, HA and FHA NPs

	Microbial strains (CFU mL^{-1}) (mean LogCount ± SD)			
Groups (n=5)	Control	BG	HA	FHA
E. faecalis	4.38 ± 0.04^{abc}	3.34 ± 0.05^{ad}	3.26 ± 0.07^{be}	3.14 ± 0.07^{cde}
S. mitis	4.31 ± 0.05^{fgh}	3.18 ± 0.08^{fi}	3.17 ± 0.07^{gj}	3.03 ± 0.07^{hij}

The same letters denote the groups which have statistically significant differences.

DCT

Table 2 and Fig 4 show the antimicrobial effects of the prepared samples in the four experimental groups of control (AH26 epoxy sealer) and the BG-modified, HA-modified, and FHA-modified sealers against the microbial strains.

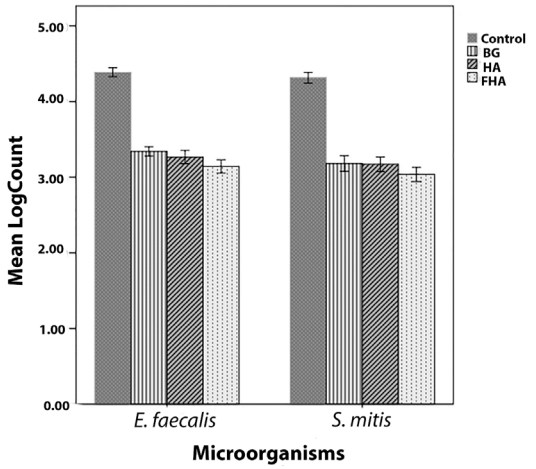

Fig 4. Viable Microorganism Counts (CFU/1- ml) of *E. faecalis* and *S. mitis* Obtained by DCT of Experimental Groups (control [conventional epoxy sealer] and sealers modified with BG NPs, HA NPs, and FHA NPs)

According to the findings, the unmodified samples exhibited the least significant antibacterial effects against *E. faecalis* and *S. mitis* compared to the modified samples (P=0.00), while the FHA-modified samples had the most significant antibacterial effects in the DCT (P>0.05). The obtained significance levels between the FHA- and BG-modified groups were estimated at 0.001 and 0.026, respectively, while they were estimated at 0.031 and 0.049 between the FHA- and HA-modified specimens, respectively. However, the antibacterial effects of the BG- and HA-modified samples on *E. faecalis* (P=0.24) and *S. mitis* (P=0.99) were not considered statistically significant.

DISCUSSION

The persistence and recolonizing of bacteria in the root canal environment play a pivotal role in the development of endodontic infections. Despite the advancement in treatment techniques and technologies (e.g., mechanical instrumentation and use of various chemical antibacterial agents), the failure rate of root canal treatments has not decreased in recent decades [28, 29]. As such, it could be assumed that the current techniques and materials are not sufficient to eliminate all the microorganisms that are present in the root canal system. Several attempts have been made to incorporate antibacterial agents into root-canal-filling materials in order to reduce the microorganisms in the root canal environment [7, 11, 12].In the present study, three synthesized calcium phosphate nano-fillers (BG NPs, HA NPs, and FHA NPs) were incorporated into the conventional epoxy-based dental sealer in order to improve its antibacterial properties. In our

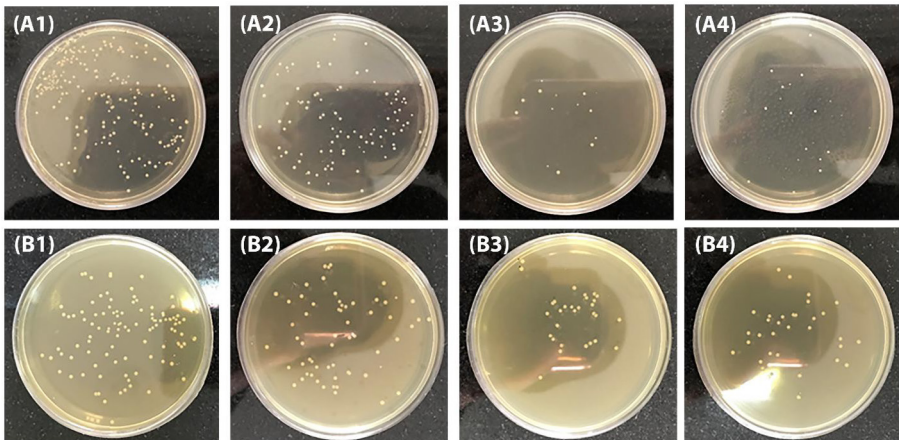

Fig 5. Image of Microbial Counts of A) *E. faecalis* and B) *S. mitis* Evaluated by DCT in A1, B1) Unmodified Specimens (control) and specimens modified with A2, B2) BG NPs, A3, B3) HA NPs, and A4, B4) FHA NPs (Number of microorganisms reduced in modified samples compared to unmodified specimens in both bacterial strains)

unpublished study, the effects of the addition of these NPs on the physical and bioactivity properties of the epoxy-based dental sealers have been described. However, the present study aimed to evaluate the effects of the mentioned NPs on antibacterial properties. The bacterial strains used in the current research were *E. faecalis* and *S. mitis*. According to the literature, *Streptococci* are among the most prevalent bacterial species that are isolated from infected root canal systems, and the most commonly known streptococcal species are *S. anginosus* and *S. mitis* [3]. The main cause of endodontic failure is persistent periapical infections, and various bacterial species have been detected in these lesions. Among these species, *E. faecalis* has been reported to be the most prevalent microorganism in teeth with periapical infections, as well as re-treatment cases [5, 6].

Several studies have evaluated the antimicrobial activity of endodontic sealer using various *in-vitro* tests, such as the agar diffusion test (ADT) [30], DCT [30, 31], and biofilm formation methods [32]. Since ADT has been reported to have numerous limitations, it is no longer recommended for the evaluation of the antibacterial activity of dental sealers [33]. Alternatively, DCT has overcome various shortcomings of ADT, thereby becoming a more reliable and reproducible quantitative test for the evaluation of antimicrobial activity in root canal sealers. In the current research, DCT was applied to assess the antibacterial properties of the added fillers. However, since DCT cannot measure antibacterial activity on established biofilms, we also evaluated the counts of the bacterial strains of the formed biofilm on the surface of the prepared specimens using biofilm formation methods.

According to the results of the present study, the incorporation of all the calcium phosphate-synthesized nano-fillers could improve antibacterial properties against *E. faecalis* and *S. mitis.* Unexpectedly, the antibacterial affects of HA NPs and FHA NPs were significantly more potent compared to the BG NPs. Furthermore, FHA displayed the most significant antibacterial activity in this regard.

The antibacterial properties of BG have been well documented [34, 35]. BG consists of SiO_2, Na_2O, CaO_2, and P_2O_5 with various concentrations, and several mechanisms have been proposed regarding its antibacterial activity. One of the proposed mechanisms for the antibacterial effects of BG is increased pH and osmotic pressure of >1% due to ion release into aqueous solutions [14, 34]. These changes could lead to cell damage and inactivation of the bacterial enzymes. Moreover, increased pH may hinder the establishment of the proton motive force for the synthesis of adenosine triphosphate (ATP) [36].

Another possible mechanism that has been identified for the antibacterial activity of BG is the Ca/P precipitation, which leads to mineralization on the surface of bacteria and destruction of the bacterial cell wall by BG debris [14, 34]. In a study in this regard, Hu et al. reported that the antibacterial properties of BG are dose-dependent and exhibited on the surface of bacteria [34]. Our unpublished study indicated that the addition of 20% FHA NPs adversely affected the physical properties of the epoxy-based sealers. Accordingly, we only used a single dose (10%) for the evaluation of the antibacterial effects of the synthesized calcium phosphate nano-fillers. Although HA alone showed no antibacterial effects in the current research, other studies have denoted that HA NPs have bactericidal activities against various microorganisms [19, 37].

At the nanometer scale, the behavior of materials differs compared to the bulk structure due to the increased ratio of the surface area to the volume, which leads to higher biological activity and chemical reactivity. The increased chemical reactivity results in the increased production of reactive oxygen species (ROS), which have potent bactericidal effects [38]. Considering these changes, nano-materials have been extensively used as antibacterial agents in dentistry in recent years [39].

Various mechanisms have been identified for the antimicrobial properties of HA NPs. The antibacterial effects of HA NPs could be attributed to the surface charge of the microorganism [37, 40]. Furthermore, gram-positive bacteria have been reported to be more susceptible to HA compared to gram-negative bacteria since gram-positive bacteria have a thicker cell wall and more peptidoglycans, which is negatively charged, while HA NPs are positively charged. As such, more calcium ions may be trapped by negatively charged peptidoglycans in case of gram-positive bacteria [37]. *E. faecalis* and *S. mitis*, which were employed in the current research, are both gram-positive bacteria. Therefore, the potent effects of the HA NPs could be attributed to the structure of

their cell membrane. In addition, the antibacterial effects of the HA NPs may be ascribed to the significant production of ROS such as OH^-, H_2O_2, and O_2^- on the surface of the HA NPs. Another plausible reason for the antibacterial affects of HA NPs could be the mechanical damage to the bacterial cell membrane by these agents [37].

In another research in this regard, Wang et al. described various mechanisms for the antibacterial properties of NPs, including ROS-induced oxidative stress, ion release, and the interaction of NPs with the cell barrier. Furthermore, the findings of the mentioned study indicated that factors such as size, shape, roughness, zeta potential, doping modifications, and environmental conditions may also influence the antibacterial mechanisms of NPs [41].

The aforementioned mechanisms for the antibacterial activity of NPs may also apply to the antibacterial effects of FHA NPs against the two bacterial species used in the present study. Moreover, fluoride could affect and interfere with the metabolism and growth of oral bacteria via numerous complex mechanisms [25]. Some of the mechanisms through which fluoride may affect and interfere with the metabolism and growth of oral bacteria include interference in the glycolytic pathway by the inhibition of metalloenzyme and enolase, direct inhibition of H+/ATPase, reduction of the cellular peptidoglycan content, and interference in glycogen synthesis, which is the principal intracellular carbohydrate storage compound synthesized by microflora such as the streptococci, lactobacilli, and actinomyces [24, 25, 42]. Furthermore, fluoride may influence specific enzymes, such as phosphatase, peroxidase, pyrophosphatase, and catalase, thereby leading to the decomposition of bacterial cell walls [42].

CONCLUSION

According to the results, the incorporation of nano-structured FHA, HA, and BG fillers into an epoxy-based dental sealer could improve its antimicrobial properties. Furthermore, the addition of the FHA NPs resulted in the most significant antibacterial effects against *E. faecalis* and *S. mitis.* Therefore, the results of this preliminary study demonstrated that nano-structured FHA, HA, and BG fillers incorporated into an epoxy-based dental sealer could be used as potentially effective biomaterials in root canal treatments.

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