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Research Article

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The effect of using nanoparticles in bioactive glass on its antimicrobial properties

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No potential conflict of interest relevant to this article was reported.

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ABSTRACT

Objectives: This study addresses the effect of using nanoparticles (np) on the antimicrobial properties of bioactive glass (BAG) when used in intracanal medicaments against *Enterococcus faecalis* (*E. faecalis*) biofilms.
Materials and Methods: *E. faecalis* biofilms, grown inside 90 root canals for 21 days, were randomly divided into 4 groups according to the antimicrobial regimen followed ($n = 20$; BAG-np, BAG, calcium hydroxide [CaOH], and saline). After 1 week, residual live bacteria were quantified in terms of colony-forming units (CFU), while dead bacteria were assessed with a confocal laser scanning microscope.
Results: Although there was a statistically significant decrease in the mean CFU value among all groups, the nano-group performed the best. The highest percentage of dead bacteria was detected in the BAG-np group, with a significant difference from the BAG group.
Conclusions: The reduction of particle size and use of a nano-form of BAG improved the antimicrobial properties of the intracanal treatment of *E. faecalis* biofilms

Keywords: Bioactive glass; Biofilm; Confocal laser scanning microscopy; Nanoparticles

INTRODUCTION

Microorganisms found within infected root canal spaces colonize as free planktonic cells or as micro-colonies joined to one another or to the root canal walls, forming biofilms. Although planktonic microorganisms can be eradicated by a variety of strategies, complete removal of biofilm bacteria from the root canal continues to be difficult [1].

Facultative Gram-positive *Enterococcus faecalis* (*E. faecalis*) is the most prevalent microorganism isolated from root canals with persistent periradicular lesions [2]. *E. faecalis* can invade dentinal tubules, remain viable inside the tubules for a prolonged period, adhere and form biofilm on dentin under various ecological conditions, resist intracanal disinfectants, and endure harsh conditions inside root-filled teeth, making root canal disinfection challenging. Disinfection is typically accomplished with chemico-mechanical procedures and antimicrobial irrigation solutions with and without intracanal medications over the course of multiple patient visits [3-5]. Mechanical treatments can considerably decrease the number of intracanal bacteria but do not totally eradicate them. Consequently, the adjunctive use of

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an intracanal medicament may help in the elimination of residual bacteria by providing an environment more conducive to periapical tissue repair [5].

Bioactive glass (BAG) was recently introduced as an intracanal medicament with positive documented results [6]. BAG consists of SiO_2, Na_2O, CaO_2, and P_2O_5 at various concentrations [7]. The antibacterial mechanism of BAG has been ascribed to a combination of factors including a high pH, an increase in osmotic pressure, and Ca/P precipitation [8].

With rapid progress in nanotechnology, nanoparticles have a promising future within the medical field. Nanoparticles are microscopic particles with at least 1 dimension measuring 1–100 nm [6]. Nanoparticles' excellent surface area, charge density, and noteworthy level of cell communication contribute to significant levels of antibacterial activity [9-11]. Additionally, the electrostatic interactions between positively charged nanoparticles and negatively charged bacterial cells results in the aggregation of numerous nanoparticles on the bacterial cell membrane and a subsequent increase in cell membrane permeability and biofilm porosity, followed by the rapid loss of membrane function [7].

Nanoparticles exhibit properties not found in their bulk equivalents; therefore, this research hypothesized that decreasing particle dimensions could significantly improve the antibacterial action of bioactive glass when used as an intracanal medicament [7]. To verify this hypothesis, this study compared the antimicrobial effect of the nano-form of BAG (BAG-np) and its corresponding bulk form (BAG) on intraradicular *E. faecalis* biofilm.

MATERIALS AND METHODS

Preparation of bioactive glass nanoparticles

Standard bioactive glass 45S5 (Perioglass™, US Biomaterials Corp., Alachua, FL, USA) was obtained from a commercial source. According to the manufacturer, this material includes 45 wt% SiO_2, 6% P_2O_5, 24.5% CaO, and 24.5% Na_2O. Bioactive glass nanopowders (BAG-np) were prepared utilizing the sol gel technique (Nano-Stream, October 6th, Egypt) [12]. The oxide composition was prepared using silicon and phosphorous alkoxides together with sodium salt (sodium hydroxide) and calcium salt (calcium hydroxide). Deionized water and ethyl alcohol were used as solvents. The gel was prepared at 70°C and pH ~ 2, aged for a week to complete the reaction, and then heat-treated at up to 800°C. The particles were analyzed utilizing transmission electron microscopy (Carl Zeiss, Oberkochen, Germany), working at 120 kV to confirm that a particle size of < 100 nm was achieved (**Figure 1**).

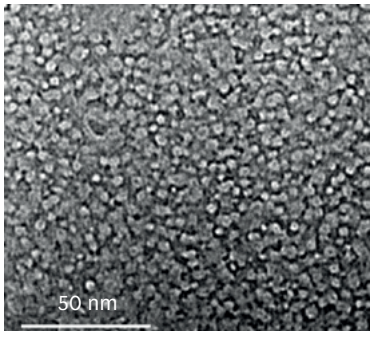

Figure 1. Transmission electron microscopy scan showing the particle size of the bioactive glass nanoparticles.

Samples preparation

Ninety human single-rooted mandibular premolars with mature apices were employed in this study. The teeth were decoronated at the point of the cementoenamel junction to standardize specimen length at 15 mm. Size 5, 4, and 3 Gates-Glidden burs were utilized to flare the coronal part of each canal. Each canal was instrumented with a size 50 Ni-Ti K-file (Dentsply Maillefer, Ballaigues, Switzerland). Abundant 2.5% sodium hypochlorite irrigation was applied throughout the instrumentation process. The canals were finally flooded with 5 mL of 17% EDTA solution for 3 minutes to remove the smear layer, followed by a final flush of 3 mL of 0.9% normal saline. The specimens were coated twice with blue nail polish, including the apical foramen, then autoclaved at 15 psi for 15 minutes at 121°C to ensure adequate sterilization. All samples were viewed under magnification to check that the nail varnish was not affected by the sterilization. Then, 5 randomly selected samples were longitudinally grooved, split, and viewed under scanning electron microscopy (SEM) (Quanta FEG 250, FEI, Hillsboro, OR, USA) to verify the sterilization.

Sample infection

A clinical reference isolate of *E. faecalis* standard strain was obtained (ATCC 29212) from the Microbiology Research Laboratory, Faculty of Medicine, Ain Shams University. A single colony was cultured on top of brain-heart infusion (BHI) agar (Land Bridge Technology Co, Ltd, Beijing, China), and then incubated anaerobically in an anaerobic container at 37°C for 1 day to obtain 1.5×10^8 colony-forming units (CFU)/mL, which is equivalent to an optical density of 0.5 at 620 nm (Bio Photometer, Eppendorf, Hamburg, Germany). Every sample was moved into a 1.5 mL Eppendorf cylinder and inoculated with 1 mL of the bacterial suspension. The cylinders were centrifuged twice for 5 minutes at 2000 × *g* to encourage bacterial infiltration with suspension substitution after each cycle. Subsequently, all samples were incubated aerobically at 37°C in BHI broth for 8 weeks. The medium was changed every 24 hours [13].

Five randomly selected samples were longitudinally grooved, split, and then washed with phosphate-buffered saline (PBS) (Lonza, Walkersville, MD, USA) for 5 minutes to remove any non-adhered planktonic microbes. These samples were viewed under SEM (Quanta FEG 250, FEI) to prove the existence of *E. faecalis* biofilms in the root canal and inside the dentinal tubules (**Figure 2**).

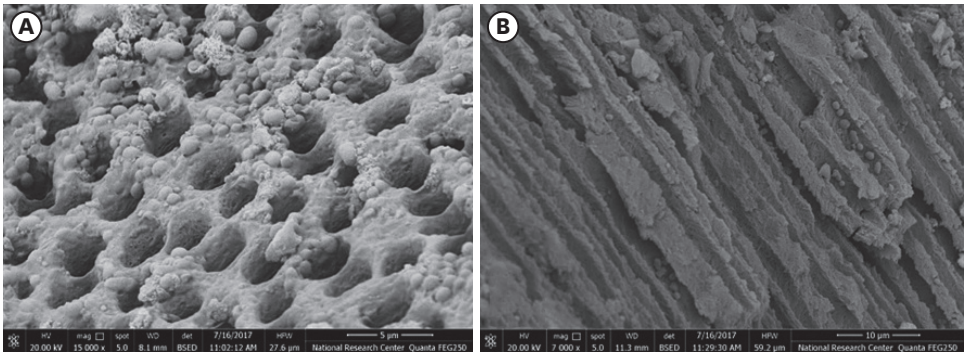

Figure 2. Scanning electron microscope images showing *Enterococcus faecalis* biofilm on (A) dentin surface and in (B) dentinal tubules after 21 days.

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Sample classification

The specimens were randomly divided into 2 experimental groups and 2 control groups (n = 20) according to the applied intracanal medicaments:

- BAG group: BAG powder was added to distilled water at the ratio of 1:1 wt/vol to obtain a homogeneous, smooth, creamy paste. This paste was introduced into the root canal with the aid of a lentulo spiral and size 40 plugger.
- BAG-np group: BAG-np powder was used as described in the BAG group.
- CaOH group (positive control): calcium hydroxide paste (Apexcal, Ivoclar Vivadent) was used as described in the BAG group.
- Saline groups (negative control): only canal irrigation was done using saline for 3 minutes.

Finally, all the specimens were coronally sealed with composite (3M ESPE, St. Paul, MN, USA), placed in microtubes, and incubated at 37ºC for 7 days.

Assessment of antibacterial activity

After one week, 10 samples from each group were opened and flooded with 20 mL of sterile saline solution to remove the root canal content. Bacteriological sampling and CFU counting protocols were followed to determine the antimicrobial efficacy in each group [10]. The canals were loaded with sterile 0.85% normal saline solution and stirred for 1 minute with a #40 H-file. Subsequently, 2 #40 pre-autoclaved paper points were introduced into the canal for 60 seconds to gather the bacteria. The paper points were transferred to 1 mL of saline, and the process was duplicated twice. All samples were mixed with a vortex mixer for 30 seconds and 10-fold dilutions were set up in saline. Aliquots of 0.1 mL were plated onto BHI agar plates, incubated for 48 hours at 37°C, and the number of CFUs per 1 mL was counted.

Confocal laser scanning microscopy assessment

The test specimens were longitudinally split, washed with PBS to remove all canal content, and then stained with acridine orange (SIGMA-ALDRICH, St. Louis, MO, USA) and propidium iodide (SERVA Electrophoresis GmbH, Heidelberg, Germany) to be observed under CLSM (LSM 710, Carl Zeiss Microscopy GmbH, Jena, Germany) at ×20 magnification. In this process, 2–3 random circular areas of 200 starting from the root canal space toward the cementum side were scanned separately. The mean intensity value of red fluorescence (dead bacteria) at the maximum intensity projection of 3-dimensional images was measured using ZEN 2012 blue edition software (Carl Zeiss Microscopy GmbH, Jena, Germany). The mean intensity value of red fluorescence to total fluorescence showed the extent of dead cells in the biofilm.

The mean and standard deviation values for CFUs and CLSM measurements were calculated. The data showed a parametric (normal) distribution by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Comparisons were carried out using 1-way analysis of variance, the post-hoc Tukey test, and the simple t-test. The statistical assessment was conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, USA) with statistical significance indicated by $p \leq 0.05$.

RESULTS

CFU test

The mean values and standard deviations of CFU counts are demonstrated in **Table 1**.
The bacterial concentration in the saline group had the highest count of microorganisms,

Table 1. The mean ± standard deviations of CFU in all groups

Variables	CFU ($\times 10^4$)/mL
Saline	10.3 ± 1.4^a
CaOH	4.4 ± 0.7^b
BAG-np	0.9 ± 0.4^c
BAG	1.7 ± 0.3^d
p value	< 0.05

Data are presented as mean ± standard deviation. Mean values followed by different lowercase letters represent statistically significant differences ($p < 0.05$).
CFU, colony-forming units; CaOH, calcium hydroxide; BAG-np, bioactive glass nanoparticles; BAG, Bioactive glass.

demonstrating that bacteria survived the test period and affirming the validity of the methodology. Although each experimental group showed a statistically significant decrease in the mean CFU count, none demonstrated total elimination of the biofilm microorganisms. Overall, the BAG-np group showed the highest antimicrobial activity, followed by the BAG group, and the lowest activity was found in the saline group, with statistically significant differences between each group ($p < 0.05$).

CLSM

Mean and standard deviation values are presented in **Table 2.** A statistically significant difference was noted among all groups, with the highest percentage of dead bacterial cells found in the BAG-np group, followed by the BAG group, with the lowest percentage in the saline group (**Figure 3**).

DISCUSSION

The complexities of the root canal system pose the main challenge to adequate disinfection in endodontics [4]. The conventional use of sodium hypochlorite as an irrigant in root canal treatment may kill microbial biofilms, but unfortunately, the limitations of this ability have been reflected in the outcomes of root canal treatment [1]. Several studies have focused on improved therapeutic plans for the disinfection of endodontic biofilms. One study involved the use of bioactive glass and another focused on the use of antibacterial nanoparticles [8,9].

There has been enthusiasm for using BAG in root canal disinfection because of its antibacterial properties, which are attributed to the increase in pH, increase in osmotic pressure, and Ca/P precipitation, which induces mineralization on the bacterial external wall [7,14].

Nanoparticles are microscopic particles with at least 1 dimension in the range of 1–100 nm. They are perceived to have properties that differ from their bulk equivalents [7]. Thus, our

Table 2. The mean ± standard deviation percentage values of dead bacteria by CLSM

Variables	Percentage of dead bacteria (red)
BAG-np	$59.9^a \pm 4.9$
BAG	$54.1^b \pm 6.8$
CaOH	$42.3^c \pm 8.9$
Saline	$34.5^d \pm 10.2$
p value	< 0.05

Data are presented as mean ± standard deviation. Mean values followed by different lowercase letters represent statistically significant differences ($p < 0.05$).
CLSM, confocal laser scanning microscopy; BAG-np, bioactive glass nanoparticles; BAG, bioactive glass; CaOH, calcium hydroxide.

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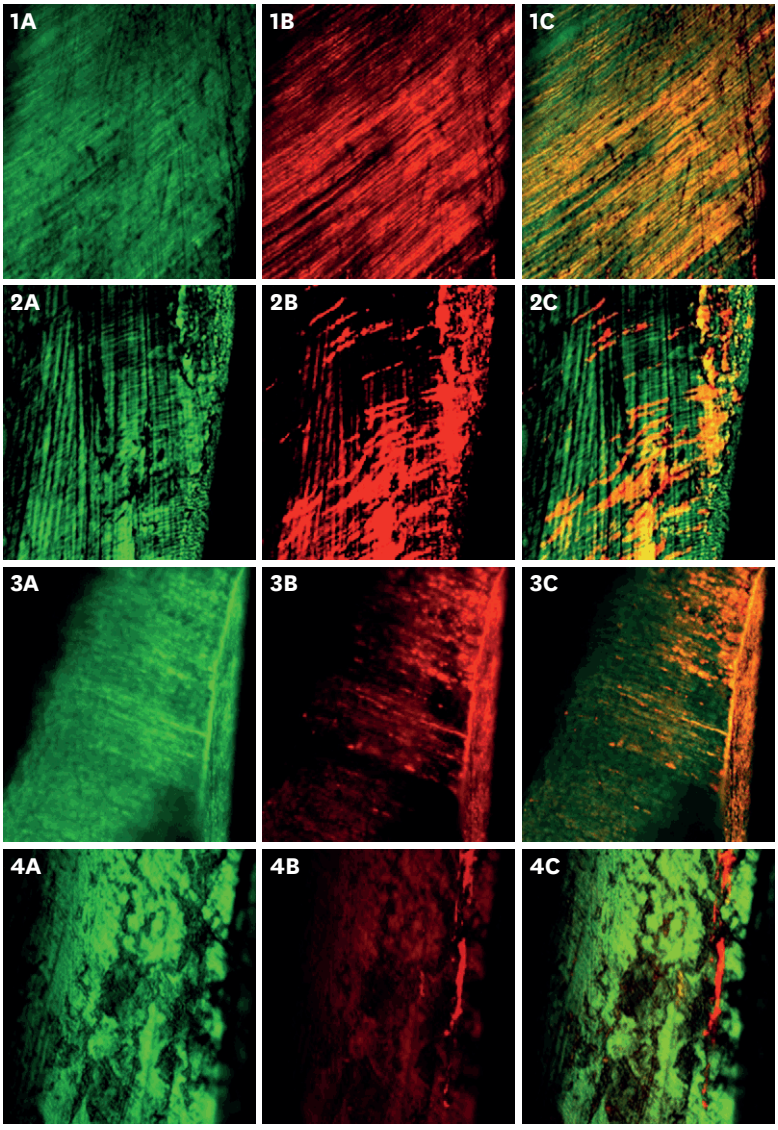

Figure 3. Confocal laser scan images of 1: bioactive glass nanoparticles, 2: bioactive glass, 3: calcium hydroxide, and 4: saline. The green fluorescence represents live bacteria, while the red fluorescence represents dead bacteria. (A) fluorescence of live bacteria, (B) fluorescence of dead bacteria, (C) A+B.

study aimed to utilize the advantages of nanotechnology to enhance the antibacterial effects of bioactive glass against intraradicular biofilm *in vitro*. This was done inside canals to better mimic clinical conditions and to include anatomical challenges, such as areas of the root canal system that cannot be reached by instruments.

E. faecalis was the preferred organism for this study due to its prevalence in persistent endodontic infections as well as its well-known resistance to root canal treatment [3]. Their small size and spherical shape also helped them to withstand the centrifugal force that was used to infect the dentinal tubules, as confirmed by SEM and CLSM observations that showed clear images of biofilm formation on canal walls and deep penetration of microorganisms into the dentinal tubules [15].

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The antimicrobial effect in this study was assessed via 2 methods: CFU testing and CLSM. In the CFU testing, residual microorganisms were gathered with sterile paper points from the root canal system [14]. This testing approach has constraints because the paper points are specific for gathering planktonic bacteria. In addition, the paper points cannot gain access to irregularities of the radicular canal system. Therefore, we accomplished bacterial sampling by instrumenting the canal wall with sterile #40 files to remove dentin, allowing more predictable sampling. CLSM was then done to overcome the limitations of the CFU test. CLSM can evaluate the outer surface as well as the deep sub-surface in the form of 3-dimensional images and can differentiate viable and dead bacteria in the same laser scanning area [15,16].

The results of this study highlighted nanoparticles as having the best antimicrobial effect, as seen in the BAG-np group, followed by the BAG group and the CaOH group. However, none of the medicaments were able to completely eradicate *E. faecalis*, confirming reports from other studies [17]. BAG has also been proven to exhibit antibacterial activity. Stoor *et al.* [8] attributed the antibacterial properties of BAG to surface reactions. Our results demonstrated that the antibacterial impact of bioactive glass was improved by decreasing its particle size, in accordance with the findings by Waltimo *et al.* [18], who attributed this improvement to the continuous discharge of alkaline species. In contrast, Balamurugan *et al.* [19] reported that bioactive gel-glass had no bactericidal impact on *Escherichia coli*. This might be explained by the larger particle size, ranging from 100 to 700 μm, as well as the subclinical concentrations that were analyzed [12].

CONCLUSIONS

The hypothesis of this study—that using smaller particle dimensions could significantly improve the antibacterial impact of BAG when used as intracanal medicaments—was confirmed. The tested nanoparticles possessed higher antibacterial properties than their bulk equivalents. Therefore, BAG-np has possible anti-biofilm capabilities and further studies with *in vivo* models should be done to validate this potential application.

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