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ANTIBACTERIAL EFFICIENCY OF ENDOSEQUENCE BIOCERAMIC SEALER INCORPORATED WITH SILVER AND CHITOSAN NANOPARTICLES (AN IN-VITRO STUDY)

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ABSTRACT

Introduction: The present study evaluated the antibacterial efficiency of Endosequence bioceramic sealer (BC) and with the addition of silver and chitosan nanoparticles (NPs).

Methods: Thirty extracted human maxillary anterior teeth were sectioned horizontally at the level of cemento-enamel junction. The teeth were randomly divided into three equal groups (10 teeth each) according to the material used; Group 1: Endosequence BC, Group 2: Endosequence BC + Silver NPs and Group 3: Endosequence BC + Chitosan NPs. Silver and chitosan NPs were synthesized and characterized then, pastes of both NPs were prepared. Root canal enlargement was performed. C linical isolate of *E. faecalis* was inoculated. Endosequence BC sealer was used according to manufacturer's instructions. Silver NP and Endosequence BC sealer were combined by adding nanoparticles of 2.3% by volume. Chitosan NP was used at 15% by volume.

Results: There was a statistically significant difference between EndoSequence BC as well as EndoSequence BC+Silver NPs groups and EndoSequence BC+Chitosan NPs group (p=0.007). No significant difference was recorded between EndoSequence BC and EndoSequence BC+Silver NPs groups (p>0.05). A significant increase in bacterial growth was observed for EndoSequence BC+Chitosan NPs (0.19±0.01), then EndoSequence BC (0.18±0.01), with least bacterial growth in EndoSequence BC+Silver NPs (0.17±0.01). All tested materials had bactericidal effect and eliminated *E. faecalis* on trypticase soy agar.

Conclusions: Addition of silver and chitosan NPs to Endosequence BC sealer did not significantly increase its anti-microbial efficacy against *E. fecalis*.

KEY WORDS: Bioceramics, Chitosan, Endodontic treatment, Nanoparticles, Silver

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INTRODUCTION

The main goal of root canal treatment is complete removal of bacteria and toxins from inside the root canal system and sterilization of the canal if possible. Presence or absence of micro-organisms in root canals during obturation plays a crucial role in the success of endodontic treatment. Removal of microbial colonization from the root canal system is performed through disinfection protocol and preventing the entry of new micro-organisms to the root canals.[1]

After pulpal infection, bacteria can invade the dentinal tubules and reach the periapical tissues therefore, conventional chemico-mechanical procedures of canal preparation are not capable of eradication of bacteria completely from root canals and dentinal tubules. Approximately 20-30% of micro-organisms can survive due to their inaccessible location in isthmuses, additional canals, and apical region.[2] In addition, common irrigation solutions used during endodontic treatment, like sodium hypochlorite, cause its effect through direct contact to the bacteria. As result, the bacteria that penetrate to the deeper layers of dentin can be survived since the depth of penetration of the irrigant is limited.[1,2]

Obturation materials and sealers with anti-microbial properties had been introduced in the market. Bioceramic (BC) sealers are now available for their bio-mineralization, bio-active and anti-microbial properties.[3]

Recently a new generation of calcium silicate based endodontic sealers has been introduced, among them is Endosequence BC sealer (Brasseler USA, Savannah. GA). EndoSequence BC sealer is premixed putty or syringe-able paste that is formed mainly of calcium silicate, calcium phosphate, zirconium oxide and tantalum oxide.[4] EndoSequence BC has been suggested as an alternative to mineral trioxide aggregate (MTA) because it is bioactive, which is defined as having the capacity to precipitate apatite-like substance upon contact with tissue fluids.[5]

Owing to the hydrophilic property, smaller particle size and low surface tension of Endosequence BC sealer, it has the ability to widely spread inside the root canal increasing its sealing ability, even to the lateral canals. Additionally, due to their high alkaline effect (+12 pH), BC sealers are characterized by their anti-microbial properties.[6]

Although many species of anaerobic bacteria have been isolated from failed root canal treatment, *Enterococcus faecalis* (*E. faecalis*) is considered to be one of the most commonly found bacteria in persistent peri-radicular lesions.[7] It was claimed to be present in around 38% of failed cases.[8]

Nanoparticles (NPs) have been introduced in the field of endodontics in the disinfection protocol during root canal treatment.[9] They can be applied directly alone in root canal disinfection or added to irrigants, intra-canal medications and even endodontic sealers.

Silver NPs are one of different types of NPs available in the market. Silver NPs are attracted to the negatively charged bacterial cell walls, causing lysis and destruction of cell wall integrity, since they are positively charged particles.[10]

Chitosan is a biopolymer that is non-toxic, biocompatible, biodegradable with favorable biological characteristics like bactericidal, anti-inflammatory, antioxidant, and healing properties[11]

The present study evaluated the antibacterial efficiency of Endosequence BC sealer either alone or after addition of silver and chitosan nanoparticles (NPs).

MATERIALS AND METHODS

Sample Size Calculation

The total sample size was determined using power analysis for a Chi-square test for comparison between three groups. The effect size (f) was 1.41. Using alpha (α) level of (5%) and Beta (β) level of

(20%) i.e. power = 80%; the minimum estimated sample size was a total of 30 specimens. Calculation was based upon the results of Wang et al.[12]

So, Total sample size was 30, and each group included 10 samples. Sample size calculation was performed using IBM®SPSS®SamplePower® Release 3.0.1.

Samples Selection

Thirty extracted human maxillary anterior teeth were collected from Misr International University teeth bank. The selected teeth were intact with mature apices, and without any fractures or cracks. Radiographs were taken bucco-lingually and mesio-distally to detect exclude internal resorption or calcification.

Samples Preparation

All teeth were disinfected using 5.25% sodium hypochlorite for 30 minutes. Any soft tissue deposits and calculus were removed and cleaned off the teeth. Then, all teeth were sectioned horizontally at the level of cemento-enamel junction using isomet saw (Isomet, Buehler; Ltd., Lake, Bluff, IL) under water coolant. The length of all roots was adjusted to 16 mm by coronal surface grinding. All samples were then stored in sterile saline at room temperature.

Samples Grouping and Randomization

Samples were randomly divided into three equal groups according to the material used (10 teeth each);

Group 1: Endosequence BC

Group 2: Endosequence BC + Silver NPs

Group 3: Endosequence BC + Chitosan NPs

Coded samples were used throughout the study to avoid possible bias. Samples of group I were coded (E) and each sample in the group was numbered from E1 to E10. Samples from group II were coded (S) and each sample in the group was numbered from S1 to S10. Samples from group III were coded (C) and each sample in the group was numbered from C1 to C10.

Preparation of the Nanoparticles

Preparation of Silver NPs

According to Wang et al., silver nanoparticles were prepared using a chemical reduction technique. A total of 3.4 g of silver nitrate (AgNO3) was mixed with 20 ml of distilled water to prepare a solution.[13,14] The polyvinylpyrrolidone (PVP) was performed by adding PVP, glucose, and sodium hydroxide to 60ml of distilled water for use as a stabilizing agent. Then the two solutions were added together at 60˚C, while being constantly mixed at 500 rpm. As silver ions were converted into Ag NPs, the solution's color gradually changed to a greyish yellow.

Silver NPs Paste

PVP with molecular weight (M.wt) = 40K was added over the silver NPs solution with concentration of 100 ppm under temperature of 30 to 40°C with vigorous mixing to have a homogenous paste.

Optical Properties

Ultraviolet–visible spectrophotometry (UV-Vis) absorption spectra were obtained on a fiber optics spectrophotometer (Fig. 1).[15]

Size and Shape

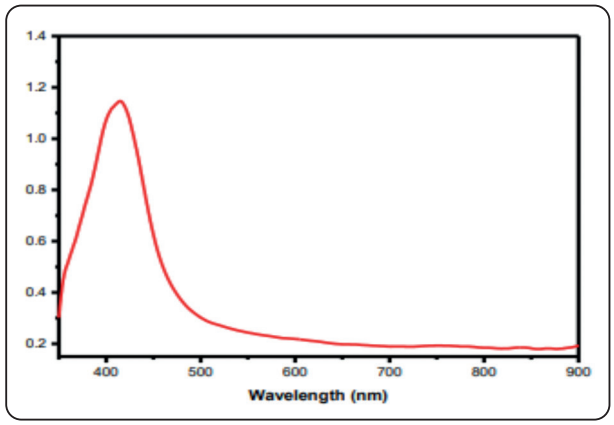

Fig. (1) Spline chart showing the Absorption spectrum of prepared silver NPs

Size and shape were confirmed using transmission electron microscope (TEM) on (JEOL JEM-2100 high resolution) and at an accelerating voltage of 200 kV (Fig. 2). Samples were prepared by adding a drop of the colloid on a copper grid, which was covered by carbon film, and the solvent was evaporated.[13,16]

Preparation of Chitosan NPs

The ionotropic gelation technique was used to prepare chitosan nanoparticles.[16] After mixing a tripolyphosphate (TPP) aqueous solution with a chitosan solution, blank NPs were produced. In a nutshell, 200 ml of 1% acetic acid (pH = 4) was used to dissolve 1gm of chitosan powder, and 400 rpm was used to stir the mixture for 6h at room temperature. Following that, 150 ml of TPP (the cross-linker) 0.2% w/v was dropped in. Then distilled water was used for cleaning the suspension with centrifuging three times at 9000 rpm for 30 minutes.

Chitosan NPs Paste

The prepared Chitosan NPs were placed in DH2O to get 10mg/ml then, PVP with M.wt= 40K was added gently and gradually over the solution under temperature of 30 to 40°C with vigorous mixing to have a homogenous paste as mentioned by earlier workers.[17]

Size and Shape

Size and shape were confirmed using TEM on (JEOL JEM-2100 high resolution) and at an accelerating voltage of 200 kV (Fig. 3). Before examination under TEM, NPs were dried under vacuum and stored in the dark at 4°C. Afterwards,

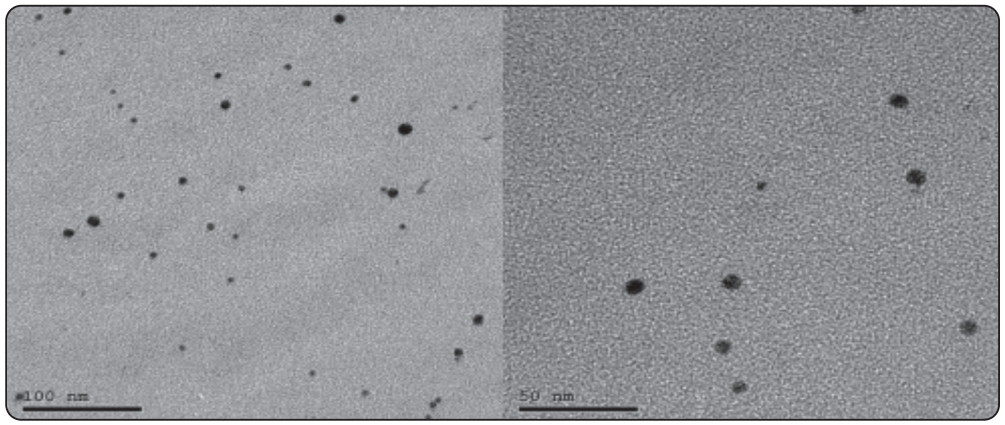

Fig. (2) TEM image of prepared silver NPs

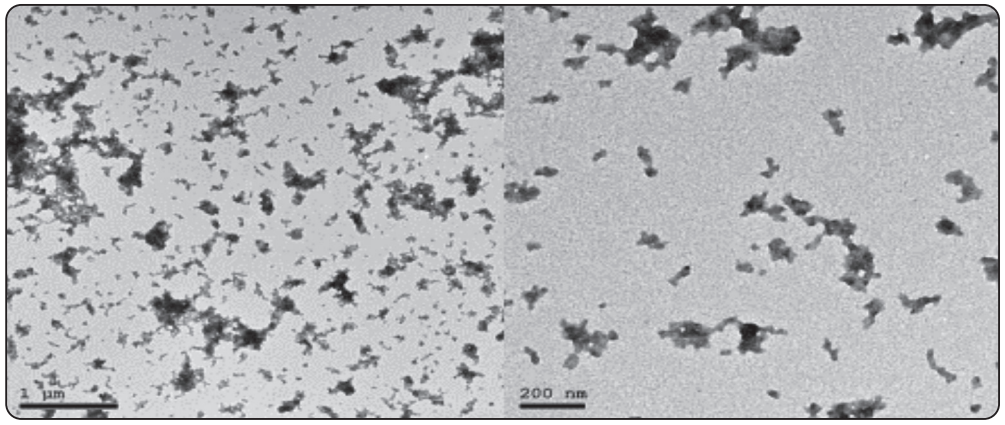

Fig. (3) TEM image of prepared chitosan NPs

TEM samples were prepared by adding a drop of NPs onto a formvar-coated copper grid, which was dried by vacuum before TEM analysis as mentioned before.[15]

Root Canal Enlargement

The orifice of the root canal was located using an endodontic explorer. A #15 Flex-o-File (Maillefer, Johnson City, TN, USA) was used for patency and determining the working length of each canal. 2ml of 5.25% sodium hypochlorite irrigant was applied between each file during all the procedural steps for all teeth, using Endo irrigation needle (27-gauge).

Cleaning and shaping was carried out using ProTaper NEXT (Maillefer, Dentsply) at the working length. Then K files were used to prepare the canals manually till reaching master apical file size #40. Finally, all teeth were rinsed with 17% EDTA solution and 2.25% NaOCl followed by sterile normal saline. Gutta-percha cone was used to manually activate each irrigant to the working length, with size #40 and taper 4%.

Bacterial Inoculation

Upon completing of the cleaning and shaping of the root canals. The teeth were sterilized by autoclaving at 121°C. Clinical isolate of *E. faecalis* (ATCC4083) from the Microbiology laboratory (Central laboratories, Ministry of Health, Egypt) was used for biofilm formation. The bacterial strain was inoculated in Brain Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 24h. The experimental suspensions were prepared by cultivating the biological marker on the surface *of* Brain Heart Infusion agar (BHIA; Difco Laboratories) following the same incubation conditions.[18] The bacterial cells were re-suspended in saline to reach a final concentration of about 3 x 10^8 cells/mL, adjusted to No. 1 MacFarland turbidity standard which was used to infect the samples (Fig. 4).

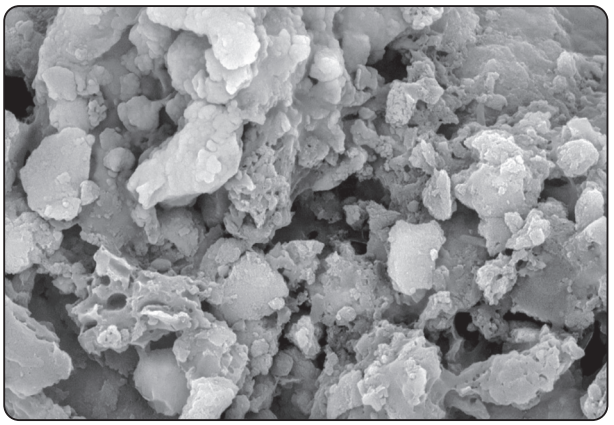

Fig. (4) SEM image of developed E. faecalis biofilm in the root canal

Preparation of the Tested Material

Endosequence BC sealer was used according to manufacturer's instructions. Silver NP and Endosequence BC sealer were combined by adding nanoparticles of 2.3% by volume.[19] Chitosan NP was used at 15% by volume.[20]

Root Canal Obturation

Paper points were used to dry the prepared canals. Master cone was adjusted to the size of the master apical file, where gutta-percha with size 40 and taper 0.04 was used. Cold lateral condensation technique was performed for obturation; by gutta-percha and the tested sealers compacted with finger spreader size 25 and accessory cones #25 taper 0.02 were applied.

Radiographs were taken for all obturated teeth at two different angulations to evaluate the obturation. The presence of any defects or voids in any sample radiographically was a sign for discarding and replacing it. Samples were then incubated for 24 hours at 37˚C for complete setting.

Evaluation of Antimicrobial Efficacy Using Direct Contact Test

Direct Contact Test

Vertical placement of 96-well micro-titer plate was done (Fig. 5). The pre-mixed Endosequence

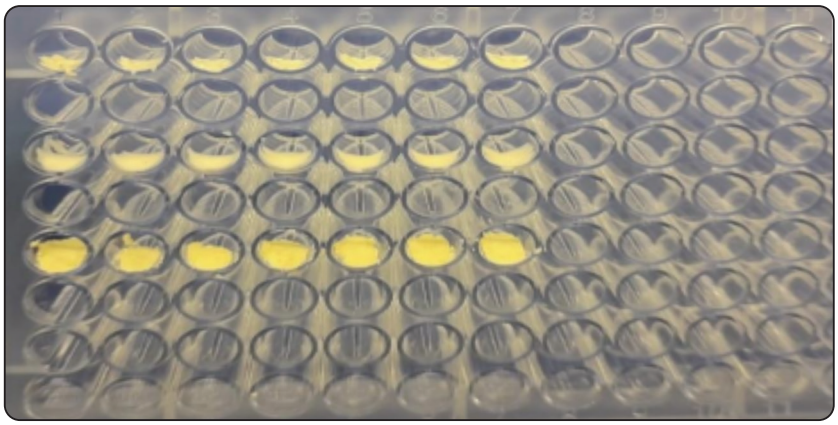

Fig. (5) An image showing vertical placement of 96-well micro-titer plate

BC sealer syringe, micro-pipette, and a cavity liner applicator were used to coat a fixed size area in the side wall of the wells with 100 μL of each freshly mixed material. The tested materials were placed in an incubator with 100% humidity to set for 7 days at 37˚C. The surface of each tested material was then covered with aliquots of 10 μL of bacterial suspension (containing approximately 1.5 x 108 bacteria/mL). Positive controls were uncoated wells with bacterial suspensions applied to their walls, while negative controls were coated wells with sealers but without bacterial suspension. Incubation of the plates was carried out at 37˚C for 60 minutes. The plates were then positioned horizontally to add 200 μL of TSB to each well. After that, 15 μL from each well was added to new wells to analyze the optical density.[21]

Spectro-photometer Evaluation

A spectro-photometer set at 620nm at 37˚C was used to measure the turbidi-dimetric for bacterial growth evaluation.[22, 23]

Colony Forming Units Evaluation

After being gently mixed with a pipette for 1 min, the bacterial suspension from each of the original wells was transferred to sterile microtiter plates and exposed to 10-fold serial dilutions in sterile saline. By culturing 10- μL aliquots of each dilution into Trypticase Soy Agar (TSA), the rate of bacterial survival was evaluated. The colony-forming units were then counted after being incubated for 24hrs at 37°C. To ensure reproducibility, samples were performed 5 times.[23]

Imaging and Analysis Processes

Imaging was performed by a digital camera mounted on biological research microscope. Images were then uploaded and by using image analysis software, the colony forming units and the percentage reduction were counted after the images were analyzed.

Percentage reduction was calculated using the following equation:

$$\text{Percentage reduction} = \frac{\text{Final colony cunt} - \text{Initial colony cunt}}{\text{Initial colony cunt}} \times 100$$

Statistical Analysis

The mean and standard deviation (SD) values of numerical data were presented. They were checked for normality by using Shapiro-Wilk test and the data distribution. Since there was parametric distribution of data, one-way ANOVA and Tukey's post hoc test was used for intergroup comparisons, also repeated measures ANOVA followed by Bonferroni post hoc test was used for intragroup comparisons. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with R statistical analysis software version 4.1.2 for Windows 1.

RESULTS

Collected data of each group are presented in table (1) and Fig. (6).

Results of contact of bacteria with sealer after 60 min

There was a statistically significant difference between EndoSequence BC as well as EndoSequence BC + Silver NPs groups and EndoSequence BC + Chitosan NPs group (p=0.007). No significant difference was recorded between EndoSequence BC and EndoSequence BC + Silver NPs groups (p>0.05). A significant increase in bacterial growth was shown in EndoSequence BC+Chitosan NPs (0.19±0.01), then EndoSequence BC (0.18±0.01), with least bacterial growth in EndoSequence BC+Silver NPs (0.17±0.01).

CFU Count

All tested materials had bactericidal effect and eliminated *E. faecalis* on trypticase soy agar. Therefore, percentage reduction equation was inapplicable (Fig. 7).

TABLE (1) Mean, Standard deviation (SD) of optical density of different types of sealers.

Measurement	Optical density (mean±SD)			
	EndoSequence BC	EndoSequence BC + Silver NPs	EndoSequence BC + Chitosan NPs	p-value
Contact of *E. Fecalis* with sealer after 60 min.	0.18±0.01^{B}	0.17±0.01^{B}	0.19±0.01^{A}	0.007 *

Different superscript letters indicate a statistically significant difference within the same horizontal row *; significant (p ≤ 0.05) ns; non-significant (p>0.05)

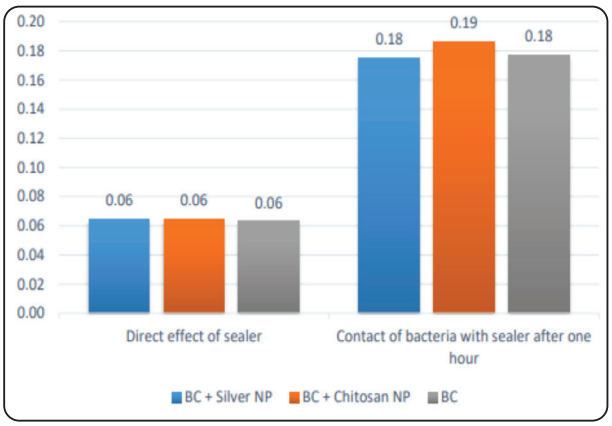

Fig. (6) Histogram showing the optical density of different types of sealers

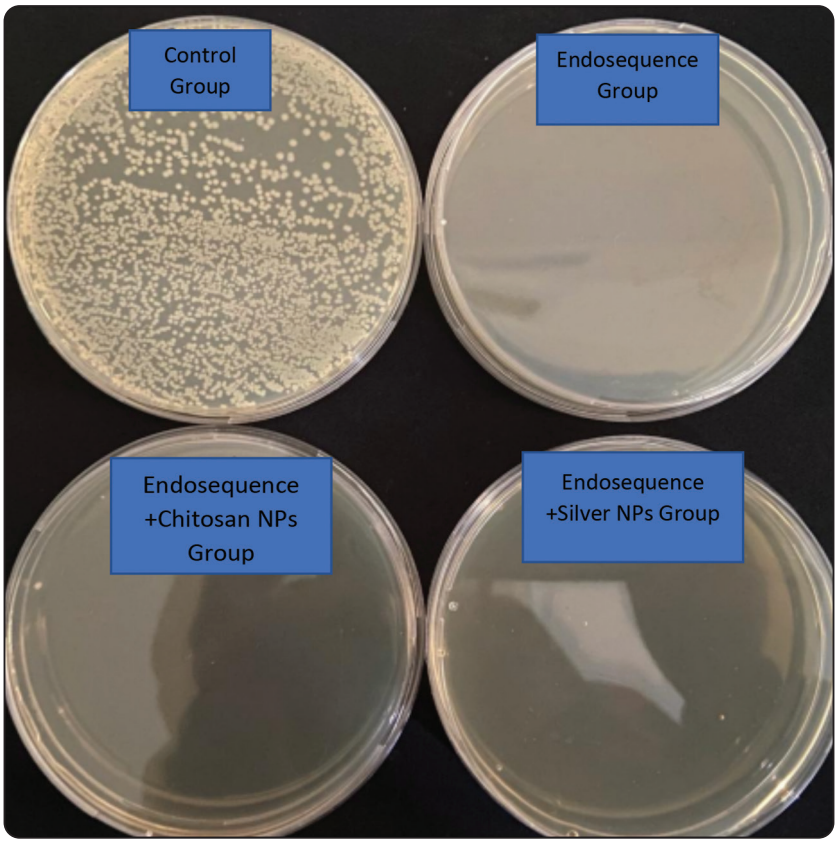

Fig. (7) An image showing all TSA plates of tested materials and control after culturing of *E. faecalis*.

DISCUSSION

For successful endodontic treatment, bacteria must be eliminated. This is hardly foreseeable, despite recent improvements in chemo-mechanical disinfection techniques. Therefore, the root canal obturation must enclose any remaining bacteria in the root canal complex and completely seal the root canal from the peri-radicular tissues.[24] Additionally, using a sealer with antimicrobial properties might further decrease the bacterial load in the root canal space.

Recently, bioceramic sealers have become widely used in endodontics. Endosequence BC sealer is one of the available BC sealers. Tricalcium silicate, dicalcium silicate, calcium phosphates, colloidal silica, and calcium hydroxide are its principal inorganic constituents.[25] This sealant is resilient in biological settings, non-toxic, and biocompatible. Because of their alkalinizing effects, it is known to have antibacterial actions. The production of calcium hydroxide and calcium silicate hydrogel may help to explain why these materials have a high pH.[26] Calcium hydroxide's constant presence assures that a high pH and antibacterial action will last for a very long time.

EndoSequence BC sealer is introduced in two different forms, involving a fast-set putty and a regular-set paste. EndoSequence BC sealer was shown to have anti-bacterial efficiency against 10 clinical strains of *E. faecalis* .[27] Furthermore, the antifungal activity of EndoSequence BC sealer and MTA was nearly similar against *C. albicans* biofilm.[28]

Nanomaterials are typically microscopic solid particles with diameters between 1 and 100 nm. Since they have large surface area and high charge density, they can attach to the surface of bacterial

cells, enhancing their antibacterial activity.[29] In dentistry, nanoparticles are either biological or metallic.

As in earlier studies, silver NPs were used in this study.[10, 30-32] When silver interacts with sulfhydryl groups in proteins and DNA, it changes the hydrogen bonding and respiratory chain, unwinds DNA, and interferes with the synthesis of cell walls and cell division, among other targets.[32] Silver, on the other hand, needs to be utilized carefully because of its concentration-dependent toxicity. There is evidence that silver NPs have harmful effects on both the environment and human health.[33] The silver NPs dispersion was biocompatible, especially at lower concentrations, according to Gomes-Filho et al in 2010.[34] So, a concentration of 23 ppm was used in this study.

The second most prevalent natural biopolymer is chitosan (poly [1, 4-b-D-glucopyranosamine], a DE acetylated derivative of chitin). Chitosan is a biopolymer that is non-toxic, biocompatible, and easily biodegraded by a variety of hydrophilic enzymes. It has beneficial biological actions such as antibacterial, anti-inflammatory, antioxidant, anticancer, and healing characteristics.[11]

The quantum-size phenomenon increases the affinity of chitosan NPs to bacterial cells. The nanoparticles strongly adsorb to the surface of the bacterial cells because of their enormous surface area, breaking the membrane and causing lysis of the bacterial cells.[20] Dental professionals have used chitosan NPs as an irrigating agent, intracanal medication, pre-treatment of the root dentin before obturation, and an ingredient in endodontic sealers.[11] As was done by Bonde et al. in 2019, chitosan NPs were added to the Endosequence BC sealer in the current investigation at a concentration of 15%.[20]

Numerous studies, including those by Barros et al. in 2014, Colombo et al. in 2018, JerezOlate et al. in 2021, and the current study, utilized the DCT.[35-37] Turbidimetric measurements are typically used to get the bacteriostatic data from DCT.[38] Using this method, the optical density of the cell can be used to calculate the rate of proliferation. This is since bacteria gradually block light entering the sample, as they grow in cell culture media.[39]

The results of the present study revealed that Endosequence BC sealer showed an enhanced antimicrobial activity. Endosequence BC sealer has a material base of MTA, zinc oxide eugenol, and bioceramic, respectively. The results also demonstrated that the antibacterial efficiency of Endosequence BC sealer's persisted for up to 30 days after setting. This antimicrobial activity of bioceramic sealers was due to its capability to release hydroxyl ions and elevate the pH values. The calcium hydroxide formed during the hydration reaction, breaks down into calcium and hydroxyl ions that accounts for the high pH observed.[40]

According to the findings of the current study, the addition of silver NPs by 2.3% by volume to Endosequence BC sealer increased the antibacterial efficacy of Endosequence BC but without significant difference. This result is coincident with the results of Garcia et al in 2016 who reported that the addition of silver NPs enhances the antimicrobial activity of both calcium silicate materials.[41] This was contributed to the ability of silver NPs to attach to the bacterial membrane, disrupting its permeability and allowing the intra-cellular components of the bacteria to come into contact with the MTA and Portland cement released ions

Mohan et al. (2020) stated that the antibacterial order was 2% silver NPs, 1% silver NPs, 2% chitosan NPs and finally 1% chitosan NPs.[42] They stated that chitosan NPs antibacterial efficiency was lower than silver NP against *E. faecalis* since chitosan is not that effective against gram-positive bacteria (*E. faecalis*).

As a result of its extremely high alkaline pH and mineral ion release, EndoSequence BC sealer exhibits its antibacterial activity during its setting

reaction. In a clinical research, photodynamic therapy was employed to sterilize the cutting surface and root-end cavity during apicoectomy procedures.

CONCLUSION

Within the limitations of the present study, we can conclude that all tested materials had bactericidal effect and addition of silver and chitosan NPs to Endosequence BC sealer did not significantly increase its antimicrobial efficacy against *E. fecalis*.

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