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Evaluation of Antimicrobial Efficacy and Adaptability to Root Canal Dentin of Bioceramic Sealer Containing Nanoparticles (In-vitro Study)

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ABSTRACT

Introduction: The aim of this study was to evaluate the antimicrobial efficacy and adaptability to root canal dentin of bioceramic sealer incorporated with nanoparticles. ***Materials and Methods:*** Silver and chitosan nanoparticles were added to Totalfill BC sealer with concentrations of 2.3 % and 15% by volume respectively. Adaptability to root dentin was evaluated using thirty single-rooted human teeth, prepared and obturated using lateral condensation technique. Samples were longitudinally sectioned and the gap area (by %) was reported using stereomicroscope x45 and scanning electron microscope x1000. The antimicrobial activity was assessed on *E. faecalis* using direct contact test after the setting of the sealer under spectrophotometer to assess the bacteriostatic activity of the tested materials. Data showed parametric distribution so one-way ANOVA followed by Tukey's post hoc test was used for intergroup comparisons and repeated measures ANOVA followed by Bonferroni post hoc test was used for intragroup comparisons. ***Results:*** The adaptability test using SEM showed that the highest gap value was found in BC + Silver NP (0.59±0.27), followed by BC (0.29±0.15), while the lowest gap value was found in BC + Chitosan NP (0.22±0.16). Post hoc pairwise comparisons showed BC + Silver NP to have a significantly higher gap value than other groups. Direct contact test showed the highest value representing highest bacterial growth was found in BC + Chitosan NP (0.19±0.01), followed by BC (0.18±0.01), while the lowest value representing lowest bacterial growth was found in BC + Silver NP (0.17±0.01). Post hoc pairwise comparisons showed BC + Chitosan NP to have a significantly higher value than other groups ($p<0.001$). ***Conclusion****:* Incorporation of nanoparticles can significantly improve the bioceramic sealer's properties.

Keywords: Antimicrobial efficacy, Adaptability, Bioceramic sealer, Nanoparticles.

INTRODUCTION

Successful endodontic treatment depends predominantly on the elimination of infecting microorganisms. This is accomplished using chemo-mechanical preparation of root canals,

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which is proven not enough and microorganisms might still survive. This is mainly due to anatomical and microbial challenges. In addition, Waltimo et al.[1] stated that despite effective cleaning and shaping of the root canals, infection may persist in 20-30% of the cases.

Grossman[2] identified the ideal sealer requirements, as having a sealer that creates hermetic seal, provides adequate adhesion with the canal wall, antimicrobial, biocompatible to periradicular tissues, and insoluble in tissue fluid. Besides, having a sealer with valuable properties like adhesion and adaptation brings two positive consequences. First, sealing of the canal will be fulfilled due to the higher sealer interface with the dentin wall.[3] Second, entombment of the remaining bacteria in dentinal tubules to prevent re-infection of periradicular tissues.[4]

A wide range of sealers are being used in endodontics; however, bioceramic (BC) sealers are gaining popularity as they possess bioactivity and biomineralization. One of the first BC sealers to be commercially available is the pre-mixed Totalfill BC sealer (FKG Dentaire, La Chaux-de-Fonds, Switzerland). Totalfill BC sealer has the same structure as Endosequence BC sealer and both have excellent physical bio compatibility properties.[5]

This sealer's small particle size, hydrophilicity and low-contact angle allow it to spread easily over the canal wall providing adaptation to dentin and also filling the lateral micro-channels.[6] BC sealers are also known to have antimicrobial properties due to their alkalinizing action. Patri et al.[7] revealed that the greatest marginal adaptation was shown by EndoSequence BC sealer, followed by ProRoot MTA sealer and then EndoREZ sealer. Poggio et al.[8] evaluated the antimicrobial efficacy of different root canal sealers including Totalfill BC against Enterococcus Faecalis (E. Faecalis) using both ADT and DCT. For the DCT on set sealers, results showed that for all time contacts, TotalFill BC Sealer was bactericidal against E. faecalis and killed all bacteria. Bronzel et al.[9] stated that TotalFill BC was significantly effective in reducing the CFU of E. faecalis when compared with AH Plus and the control.

Nanoparticles (NP) have been extensively studied in the medical field. NP have the ability to deliver drugs to the target sites and can be used in targeted therapies in treatment of different types of cancer. In the endodontic field, NP have been used in the disinfection process to reduce treatment failure.[10] They have been used alone and in conjunction with irrigation materials, intra

canal medicaments and endodontic sealers. There are different types of NP available, including metallic NP and organic NP.

Silver NP is one of the metallic nanoparticles. Silver NP are positively charged and so they interact with the negatively charged bacterial cell walls, adhere, penetrate into the bacterial cell leading to loss of cell wall integrity and permeability.[11] On the other hand, chitosan is a non-toxic, biocompatible biopolymer, which can easily be biodegraded through different hydrophilic enzymes, providing positive biological effects such as bactericidal, anti-inflammatory, antioxidant, antitumor, and healing properties.[12]

Many species of anaerobic bacteria were encountered in failed root canal treatment; however, E. faecalis is the most frequently perceived in persistent periradicular lesions.[13] According to Sundqvist et al.[14] 1998, E. faecalis found in 38% of failed root canal system.

In the current study, we evaluated the adaptability and antimicrobial efficacy of bioceramic sealer alone and after incorporation of silver nanoparticles or chitosan nanoparticles. The null hypothesis was that there is no significant difference between the tested materials in terms of adaptation to canal dentin walls and antimicrobial action.

MATERIALS AND METHOD

A. Adaptability to root canal dentin test evaluated via; Scanning Electron Microscope and Stereomicroscope: Thirty extracted anterior single rooted teeth with single canal were collected from MIU teeth bank to be uses in this study. All teeth were decontaminated by immersion in 5.25% NaOCl for 5 min.

They were, then cleaned using ultrasonic scaler to remove any surface deposits and/or calculus. Later, teeth were decapitated at the level of cement-enamel junction using isomet saw under water coolant.

Sample size calculation: This power analysis is for a one-way fixed effects analysis of variance with 3 levels. Power analysis was based upon the results of Wang et al.[15] and the estimation that Nanoparticles will provide 10% increase in antibacterial effect. The effect size f = 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 samples (10 samples per group). Sample size calculation was performed using IBM®SPSS®SamplePower® Release 3.0.1.

Sample grouping and randomization: The

teeth were randomly divided into 3 experimental groups, each containing 10 teeth according to the tested materials as follows:

Group I (n=10): Totalfill BC + Silver NP

Group II (n=10): Totalfill BC + Chitosan NP

Group III (n=10): Totalfill BC

Preparation of silver NP: Silver nanoparticles was prepared using a chemical reduction method as reported by Wang et al.[16,17] A solution of silver nitrate (AgNO3) was prepared by adding 3.4 g to 20 ml distilled water. The polyvinylpyrrolidone (PVP) has been used as stabilizing agent and was prepared by dissolving PVP, glucose and sodium hydroxide in 60 ml distilled water. Both solutions are then added together drop by drop at 60 ◦C at constant stirring 500rpm. The color of the solution slowly turned into grayish yellow, indicating the reduction of the silver ions to Ag nanoparticles.

Preparation of Chitosan NP: Chitosan nanoparticles were prepared according to the ionotropic gelation process.[18] Blank nanoparticles were obtained upon the addition of a tripolyphosphate (TPP) aqueous solution to a Chitosan solution. Briefly, 1gm of Chitosan powder was dissolved in 200ml 1% acetic acid (pH = 4) and stirred for 6h to get homogenous solution at speed 400 rpm at room temperature. Then, 150ml of TPP (the cross-linker) 0.2% w/v dropwise was added. The clear solution turned to turbid indicating formation of chitosan NP. After that the suspension was washed by centrifugation for 30min at 9000 rpm three times with distilled water (DH_2O).

Cleaning and shaping: Patency and working length were determined by passing the #15 K file to the anatomical foramen. Then length was recorded and the final working length was established 1 mm short of this recoded length. The irrigating solution that was used throughout the procedural steps was NaOCl 5.25%, delivered with a 27-gauge needle, 2 ml between each file size. The canals were cleaned and shaped by M Pro rotary NiTi files at the working length. Later on, canals were prepared manually using K files up to master apical file size #40. Finally, teeth samples received a flush with 5 ml of 17% aqueous solution EDTA chelating agent for removal of smear layer and 5 ml of 2.25% NaOCl followed by final rinse with 5 ml of sterile normal saline. Each irrigant was activated manually to the working length using gutta-percha cone #40 taper 4%. The frequency of activation used was 100 strokes per minute. Four gutta-percha cones were used per canal.

Preparation of the tested materials: The tested endodontic sealer used in this study is the premixed Totalfill BC sealer, used according to manufacturer's

recommendations. Nanoparticles and Totalfill BC sealer were incorporated using the calibrated syringe of the sealer and a micropipette to add the nanoparticles in the predetermined concentration as follows:

- o Silver NPs was incorporated at a concentration of 2.3% by volume.[19]
- o Chitosan NP was incorporated at a concentration of 15% by volume.[20]

Root canal obturation: The instrumented canals were dried with paper points. Mastercone adjustment was done using gutta-percha size 40 taper 0.04 corresponding to master apical file size, inserted to full working length and checked both visually and radiographically. Obturation was done using cold lateral condensation technique through using finger spreader size 30 and being filled with additional accessory cone #25 taper 0.02. All obturated teeth were radiographed in two different angulations to verify the adequacy of the obturation. If any defects or voids are seen on a radiograph, the sample was discarded and replaced. Specimens were then allowed to set in 100% humidity at 37˚C in an incubator for a day (24 hours) to ensure its complete setting.

Stereomicroscope evaluation: All the imaging and analysis processes were performed in Image Analysis Unit, Faculty of Oral and Dental Medicine, Misr International University. Longitudinal sectioning of the root specimens was done by first preparing a groove using high speed tapered round ended stone and then the separation was done using chisel. Each root length was measured under microscope at magnification x45 and divided into cervical, middle and apical thirds. Then images were transferred to a computer system for image analysis.

Scanning electron microscope (SEM): Root samples already longitudinally sectioned for stereo microscope were used again in the SEM analysis at x1000 magnification.[21] Specimens were placed in a chamber in a tightly controlled gaseous atmosphere and viewed without prior drying and coating under environmental scanning electron microscope (ESEM). Images were then analyzed using image J software, for calculating percentage of gaps surface area, and then converted into numerical value.

Image analysis: Analysis process was established using image J software. First, images were automatically corrected in terms of contrast and brightness. Then root canal area was selected and cut from the image. Images were then transferred to 8-bit monochrome image. Gap area at the interface between the endodontic filling and the root dentin was highlighted using color code thresholding, and a binary image was

produced for the desired area of gap before calculation. The gap area was calculated and then fractionized as a percentage to the total area of the canal.

B. Antimicrobial efficacy test evaluated via Direct Contact Test.

Bacterial inoculation:[22] The Enterococcus faecalis strain selected in this study was from the American Type Culture Collection (ATCC 4083). E. faecalis strain was grown overnight at 37 °C in tryptic soy broth (TSB) supplemented with 1% glucose. Purity of culture was checked and inoculum was adjusted in PBS to a turbidity of 0.5 McFarland scale.

Direct contact test:[23] A 96-well microtiter plate was placed vertically. An area of fixed size in the side wall of the wells were coated with 100 µL of each freshly mixed material using the premixed Totalfill BC sealer syringe, micropipette and a cavity liner applicator. The tested materials were allowed to set for 7 days at 37˚C in an incubator in 100% humidity. Then Aliquots of 10 µL of bacterial suspension (containing approximately 1.5 x 108 bacteria/mL) were then placed on the surface of each tested material. Bacterial suspensions applied to the wall of uncoated wells acted as positive control, while those coated with sealers but without bacterial suspension were negative controls. Plates were then incubated at 37˚C in 100% relative humidity for 60 min. Next, the plates were horizontally placed to permit 200 µL of TSB to be added to each well. Afterwards, 15 µL from each well was transferred to new wells for the optical density analysis.

Spectrophotometer evaluation:[24,25] Turbidimetric determination of bacterial growth kinetics was monitored in each well using a spectrophotometer at 620nm at 37˚C.

RESULTS

Adaptability to root canal dentin: Stereomicroscope:

****Impact of root third on adaptability of the sealer:** Mean, Standard deviation (SD) values of percentage of gap area of the canal (Stereomicroscope) for different sealer types within each root third are shown in **table (1) figure (1).**

****Total gap area percentage within each root:** Mean, Standard deviation (SD) values of percentage of gap area of the canal for different sealer types within each root third are shown in **table (2) figure (2)**.

Scanning electron microscope:

****Impact of root third on adaptability of the sealer:** Mean, Standard deviation (SD) values of percentage of gap area of the canal (SEM) for different sealer types within each root third are shown in **table (3) figure (3).**

Table (1): Statistical analysis of gap area percentage for different sealer types within each specimen third.

Specimen third	Gap area of the canal (%) (Stereomicroscope) (mean±SD)			p-value
	BC + Silver NP	BC + Chitosan NP	BC	
Coronal	5.20±0.06^{A}	0.90±0.01^{C}	1.77±0.02^{B}	**<0.001***
Middle	3.09±0.05^{A}	0.74±0.02^{C}	1.42±0.02^{B}	**<0.001***
Apical	2.16±0.15^{A}	0.64±0.03^{B}	0.71±0.02^{B}	**<0.001***

Different superscript letters indicate a statistically significant difference within the same row.
**= significant ($p \leq 0.05$)*

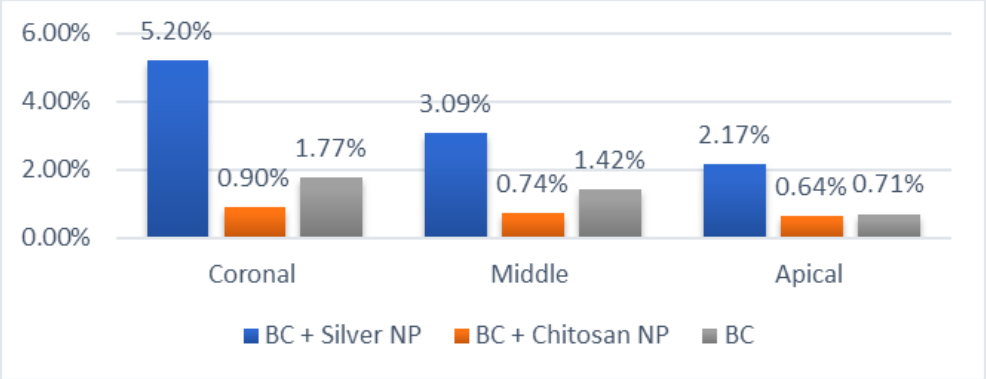

Figure (1): Bar chart showing comparison of average gap area (%) for different types of sealers at different thirds.

Table (2): Statistical analysis of gap area percentage for different sealer types.

Gap area of the canal (%) (Stereomicroscope) (mean±SD)			p-value
BC + Silver NP	BC + Chitosan NP	BC	
3.48±1.30^{A}	0.76±0.11^{C}	1.30±0.45^{B}	**<0.001***

Different superscript letters indicate a statistically significant difference within the same row.
**= significant ($p \leq 0.05$)*

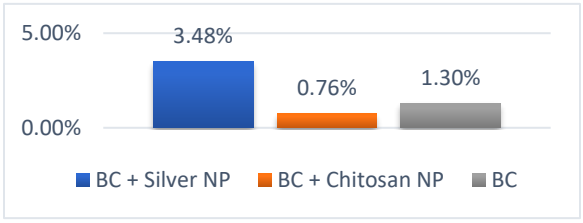

Figure (2): Bar chart showing comparison of average gap area (%) for different types of sealers at different thirds.

****Total gap area percentage within each root:** Mean, Standard deviation (SD) values of gap area of the canal (%) (SEM) for different sealer types are shown in **table (4) figure (4)**.

****Antimicrobial efficacy (Spectrophotometer evaluation):** Mean, Standard deviation (SD) values of optical density for different sealer types are shown in **table (5) figure (5)**.

Table (3): Statistical analysis of gap area percentage for different sealer types within each specimen third.

Root third	Gap area of the canal (%) (SEM) (mean±SD)			p-value
	BC + Silver NP	BC + Chitosan NP	BC	
Coronal	0.96 ± 0.02^{A}	0.43 ± 0.01^{C}	0.50 ± 0.01^{B}	**<0.001***
Middle	0.34 ± 0.01^{A}	0.18 ± 0.01^{C}	0.20 ± 0.01^{B}	**<0.001***
Apical	0.48 ± 0.01^{A}	0.06 ± 0.01^{C}	0.17 ± 0.01^{B}	**<0.001***

Different superscript letters indicate a statistically significant difference within the same row.
**= significant ($p \leq 0.05$)*

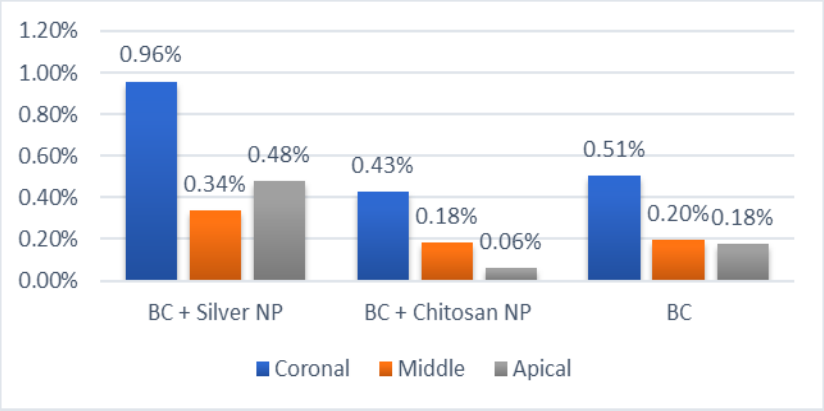

Figure (3): Bar chart showing comparison of average gap area (%) for different types of sealers at different thirds.

Table (4): Statistical analysis of gap area percentage for different sealer types.

Gap area of the canal (%) (SEM) (mean±SD)			p-value
BC + Silver NP	BC + Chitosan NP	BC	
0.59 ± 0.27^{A}	0.22 ± 0.16^{B}	0.29 ± 0.15^{B}	**<0.001***

Different superscript letters indicate a statistically significant difference within the same horizontal row.
**= significant ($p \leq 0.05$)*

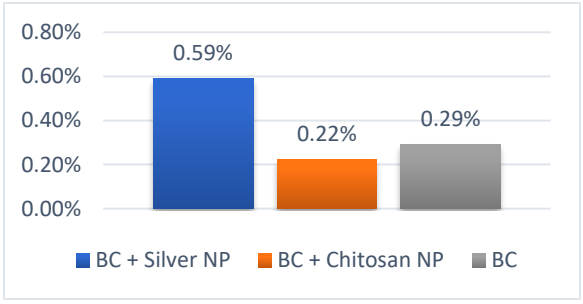

Figure (4): Bar chart showing average gap area of the canal (%) (SEM) for different types of sealers.

DISCUSSION

Bioceramic sealers are gaining a massive popularity recently in the endodontic field. Totalfill BC sealer (FKG Dentaire, La Chaux-de-Fonds, Switzerland) are among the BC sealers available in the market. Its main inorganic components include tricalcium

Table (5): Statistical analysis values of optical density for different sealer types.

Measurement	Optical density (mean±SD)			p-value
	BC + Silver NP	BC + Chitosan NP	BC	
Contact of bacteria with sealer after 60 min.	0.17±0.01 B	0.19±0.01 A	0.18±0.01 B	**0.007***

Different superscript letters indicate a statistically significant difference within the same horizontal row.
**= significant ($p \leq 0.05$)*

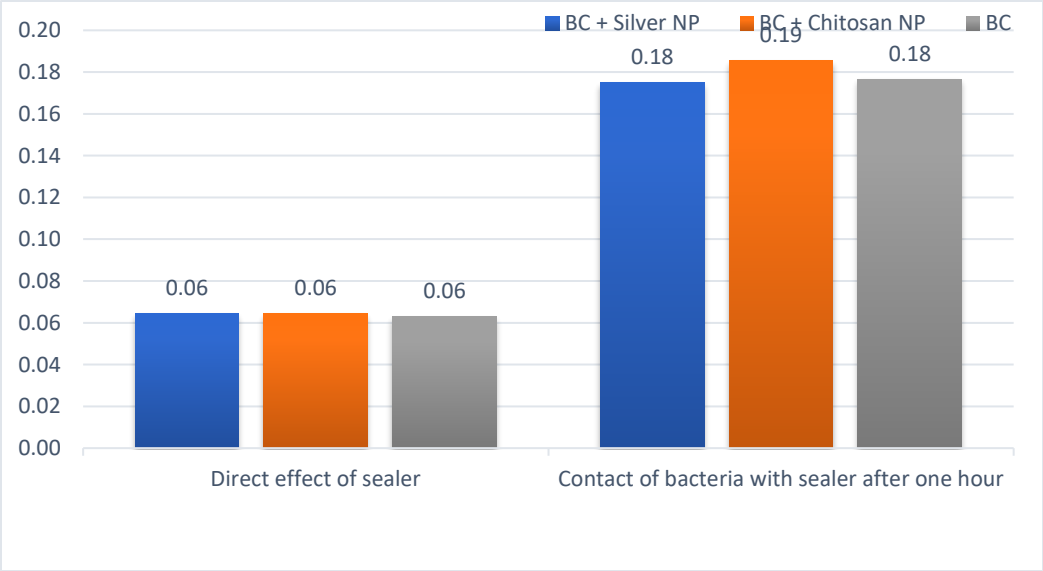

Figure (5): Bar chart showing average optical density at 620 nm for different types of sealers within each measurement.

silicate, dicalcium silicate, calcium phosphates, colloidal silica and calcium hydroxide.[26] This sealer is biocompatible, non-toxic and stable in biological environments. It's known to have antimicrobial properties due to its alkalinizing action. The high pH of such materials may be explained by the formation of calcium hydroxide and calcium silicate hydrogel.[27] The continued presence of calcium hydroxide ensures the persistence of a high pH and antimicrobial activity for a long period of time.[7,9]

Moreover, Totalfill BC sealer utilizes the inside moisture of dentinal tubules for setting. After complete setting of the material, the calcium silicate component helps creating a calcium silicate hydrate gel and calcium hydroxide. Then the calcium hydroxide interacts with the dentinal phosphate ions

forming hydroxyapatite and water. It also provides 0.20 percent growth which leads to gapless chemical bond between the sealer and dentinal walls.[28]

Nanomaterials in general are small solid particles with a diameter ranging from 1-100 nm. Their large surface area and high charge density enable them to interact with the negatively-charged surface of bacterial cells resulting in enhanced antimicrobial activity.[29] Nanoparticles used in dentistry are either metallic or organic.

Silver NP is the type used in the current study as in the previous studies by Haghgoo et al.,[30] Halkai et al.[11] and Leng et al.[31] Silver is known for its antimicrobial action as it acts on multiple targets starting from interaction with the sulfhydryl groups of proteins and DNA, alteration of the hydrogen bonding/respiratory chain, unwinding DNA, and interference with cell wall synthesis/cell division.[32] On the other hand, silver should be used with caution as its toxicity is concentration-dependent. Evidence ensures adverse effects of silver NP on both the human health and the environment.[33] According to Gomes-Filho et al.[19] silver NP dispersion was biocompatible especially in a lower concentration. Thus, 23 ppm concentration was used in the current study. Chitosan is a natural biopolymer. Chitosan NP offers great affinity to bacterial cells due to the quantum-size effect. Because of its large surface area, the nanoparticles could be tightly adsorbed onto the surface of the bacterial cells, disrupting the membrane, which would lead to the leakage of intracellular components, thus killing the bacterial cells.[20] In the current study, chitosan NP was incorporated with the Totalfill BC sealer at a concentration of 15% as done by Bonde et al.[20]

The adaptation of sealant to dentin has generally been evaluated by several methods over time. In the present study, stereomicroscope was used as done by many researchers like Akman et al.,[34] Al Hadad et al.[35] and Palanivelu et al.[36] Stereomicroscope is a reproducible and reliable method for measuring the gap areas with magnification up to x45.

SEM was used in many studies like Punithia et al.,[37] Mohammadian et al.,[26] Asawaworant et al.[38] and Patri et al.[7] SEM provides greater resolution, better interface magnification up to x2000 and higher depth of field as the SEM uses electro-magnets rather than lenses offering more control over the degree of magnification and so providing clearer images. The observation of the interface between root filling material and the dentin canal walls can be done with either

longitudinal or transverse sections. In most studies, including the present one, longitudinal sections are made particularly if the coronal and middle root canal thirds are being evaluated.[39–41] In the current study, the adaptability of BC sealer to root dentin increased in the coronal to apical direction. This result is comparable with results from several studies.[42,43] They explained that the root canal anatomy is dynamic, always changing depending on the level of the root third. Therefore, this morphological variation may lead to voids in coronal more than middle and apical thirds.[43]

Apical adaptability to dentin was superior than coronal adaptability in all groups, and it might also be due to the use of manual dynamic activation technique. This activation technique might have increased the sealer penetration apically after effective smear layer removal. The smear layer removal was established due to the push-pull motion of a well-fitting gutta-percha cone overcoming the vapor lock effect apically, leading to significantly higher apical seal as presented by Topcuoglu et al.[44]

This study demonstrated that the addition of chitosan NP to Totalfill BC sealer showed the highest adaptability to root dentin compared to both Totalfill BC alone and with silver NP, under both stereomicroscope and SEM. This result is consistent with several studies.[45,46] They explained that chitosan NP is hydrophilic, so it can come in close contact with root canal dentin increasing the wetting ability. Besides, chitosan has a number of hydroxyl and amino groups making chitosan cationic, allowing ionic interactions with the dentin calcium ions. Moreover, the amino group in chitosan can be protonated, allowing adsorption into the root dentin and dentinal tubule penetration.[47]

In the current study, silver NP mixed with Totalfill BC sealer recorded the largest mean of gap size compared to other groups under both stereomicroscope and SEM. This result was consistent with other studies.[48,49] Addition of silver NP may have decreased the flow of Totalfill BC sealer affecting its adaptability to root dentin. According to Teixeira et al.[50] the flow of AH Plus and Endofill sealers decreased with the increase of the concentration of silver NP (0%, 2.5%, 5% and 10%).

Considering the interfacial gaps between root filling materials and dentin, Totalfill BC sealer alone presented lower mean of gap values compared to Totalfill BC when mixed with silver NP. This result was consistent with several studies.[6,51] This could be explained by the alkaline caustic effect of the calcium silicate sealer's hydration products. These

byproducts have proven to degrade the collagenous component of the interfacial dentin, facilitating the penetration into the dentinal tubules.[51]

Bacterial elimination is crucial for the success of root canal treatment. However, even with the recent advances in the protocols of chemo-mechanical disinfection, this is hardly predictable. Therefore, the root canal obturation needs to entomb residual bacteria present in the root canal complex and prevent communication between periradicular tissues and oral cavity.[52] Besides, having a sealer with antimicrobial properties can help in further reduction of the bacteria present in the root canal space.

The DCT was used in many studies such as; Barros et al.,[23] Poggio et al.,[8] Colombo et al.,[53] Jerez-Olate et al.[54] and in the current study as well. The bacteriostatic information is usually gathered from DCT using turbidimetric measurements.[25] By using this technique, the rate of proliferation can be estimated by measuring the cell's optical density. That's because, bacteria proliferate in cell culture media, and their cell bodies increasingly block light that passes through the sample.[55]

The results of this study revealed that the addition of silver NP by 2.3% by volume to Totalfill BC sealer and Totalfill BC alone, were equally bacteriostatic and both were significantly better than when Totalfill BC was mixed with chitosan NP by 15% by volume. Alsubait et al.[56] described the antimicrobial activity of Totalfill BC sealer to be based mainly on its ability to release hydroxyl ions and to raise the pH values. During the hydration reaction, calcium hydroxide is formed and then dissociates into calcium and hydroxyl ions which is the reason for the high pH encountered. This study also showed that Totalfill BC sealer's antimicrobial activity lasts up to 30 days after setting.

Garcia et al.[57] reported that the addition of silver NP promoted the antimicrobial activity of white MTA. They explained that silver NP can adhere to the bacterial membrane, affecting its permeability and eventually facilitating the contact between the intracellular components of the bacteria and the ions released from the MTA and PC.

Mohan et al.[58] explained that chitosan NP's antimicrobial activity was less than silver NP against E. faecalis due to the fact that chitosan is more effective against gram-negative compared to gram-positive bacteria (E. faecalis). Therefore, the null hypothesis was rejected as silver NP had a positive effect on the antimicrobial efficacy of Totalfill BC sealer and negative one on its adaptability to

root dentin. On the other hand, chitosan increased the adaptability of Totalfill BC sealer significantly and significantly decreased its antimicrobial efficacy.

CONCLUSION

Within the limitations of the present study, it can be concluded that:

1. Addition of silver NP to Totalfill BC sealer significantly increases the antimicrobial efficacy against E. faecalis, however it negatively impacts its adaptability to root dentin.

2. Addition of chitosan NP to Totalfill BC sealer improves its adaptability to root dentin, however it negatively affected its antimicrobial efficacy against E. faecalis.

3. Apical thirds have the best adaptability to root dentin.

4. The increase in magnification from x45 to x1000 did not provide additional information about adaptability of sealers to root dentin.

CLINICAL RELEVANCE

Microorganisms are the main etiological factor of endodontic infections. Therefore, using endodontic sealers in conjunction with antibacterial agents, such as silver nanoparticles and chitosan may result in higher antibacterial efficacy and thus improve the clinical outcome (success rate) of endodontic treatment.

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