Original Article

Antibacterial Efficacy of Biosynthesized Silver Nanoparticles against *Enterococcus faecalis* Biofilm: An *in vitro* Study

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Abstract

Aim: This study aims to evaluate the antibacterial efficacy of biosynthesized silver nanoparticles (AgNPs) produced using the fungi against *Enterococcus faecalis* biofilm model on root dentin. **Materials and Methods:** AgNPs were biosynthesized using the fungi *Fusarium semitectum* isolated from healthy leaves of *Withania somnifera.* Minimum inhibitory concentration (MIC) of AgNPs was determined by microbroth dilution method using series of dilutions. MIC dose was standardized to evaluate the antibacterial efficacy. For biofilm model, thirty root dentin blocks prepared using human extracted single-rooted teeth were inoculated with *E. faecalis* in Trypticase soy agar broth for 2 weeks with alternate day replenishment and randomly divided into three groups (n = 10 each) and treated as: Group I: Sterile distilled water, Group II: AgNPs, and Group III: 2% chlorhexidine gluconate (CHX) and incubated at 37°C for 24 h. Each dentin block was rinsed in saline, vortex shaken for 60 s, and serial decimal dilutions were prepared and plated on trypticase soy agar plates and incubated for 24 h followed by CFU colony counting and statistically analyzed using one-way ANOVA followed by *post hoc* Tukey honestly significant difference test. **Results:** MIC of AgNPs for *E. faecalis* was determined as 30 mg/ml. No significant difference was seen between AgNPs and 2% CHX when compared to the control group with mean colony counts being 2.4, 2.5, and 6.77 CFU/ml (10^7), respectively ($P < 0.0001$), against *E. faecalis* biofilm. **Conclusion:** Biosynthesized AgNPs exhibit effective antimicrobial activity against *E. faecalis* biofilm on root dentin. Therefore, it can be employed as antimicrobial agent for root canal disinfection.

Keywords: *Antimicrobial agent, biofilm, endodontic disinfection, endophytic fungi, resistant microbe*

Introduction

The main etiological factors for pulp and periradicular infections are the microorganisms.[1] The main objective of the root canal treatment is the complete eradication of the microorganisms and thorough disinfection of the root canal system; however, bacteria residing within the dentinal tubules are inaccessible to currently available root canal instrumentation techniques, root canal irrigants, intracanal medicaments, and obturating materials.[2] This is because endodontic infection is a biofilm-mediated infection and complete eradication of the microbes is difficult owing to the complex anatomy of the root canal system and growing microbial resistance to the available treatment modalities. All these factors cause persistence of bacteria within root canal system leading to persistent infections and reinfections.[3,4]

The biofilm mode of the bacteria allows to survive even in nutrient-depleted environment such as root-filled teeth and shows higher antimicrobial resistance due to the protective barrier provided by the extracellular polymeric matrix (EPM) of the biofilm.[4] Therefore, antimicrobial agents which possess the ability to disrupt the EPM would penetrate into the biofilm and eliminate the bacteria. Silver nanoparticles (AgNPs) have gained popularity because of their unique ability to penetrate tissues, interact with bacteria, exhibit potent antimicrobial activity and biocompatibility.[5]

AgNPs can be synthesized by different methods. Due to the toxicity involved in the process of synthesis, biosynthesis of AgNPs using the biological modalities such as bacteria, fungi, and plants which do not produce toxic substances in their process of synthesis has gained popularity in the field of nanotechnology.[6] Among them, fungi

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have many advantages as they need simple nutrients to grow and cover large surface areas, economical and simple process for AgNPs synthesis over a range of sizes.[7]

Enterococcus faecalis is the most prominent microorganism involved in persistent infections after root canal therapy.[8] *E. faecalis* has the ability to penetrate the dentinal tubules and cementum. It can survive in biofilm form at anatomical complexities of root canal system, over the foreign bodies like gutta percha or other obturating materials extending into periapical tissues and can survive for prolonged periods under nutrient-depleted conditions.[2,9] *E. faecalis* adheres to the dental tissues by the collagen-binding proteins: angiotensin-converting enzyme and serine protease.[10] The aim of the present study is to investigate the antibacterial efficacy of biosynthesized AgNPs produced using endophytic fungi *Fusarium semitectum* against *E. faecalis* biofilm formed on root dentin models.

Materials and Methods

The present *in vitro* study was conducted in Department of Conservative Dentistry and Endodontics, HKES's Institute of Dental Sciences and Research, Kalaburagi and antibacterial studies conducted at Department of Microbiology, Gulbarga University, Kalaburagi, India.

AgNPs were biosynthesized using the endophytic fungi *F. semitectum* isolated from healthy leaves of *Withania somnifera* collected from Department of Botany, Gulbarga University, Kalaburagi and confirmed by different characterization techniques as described in our previous study.[11] In brief, cut leaf segments of *W. somnifera* were sterilized and incubated in PDA plates for 5–6 days for the fungi to grow. The fungi were isolated and identified based on morphological and microscopic characteristics. Fresh cultures of fungi were inoculated in Erlenmeyer flask containing 100 ml malt glucose yeast peptone broth and incubated at 29ºC for 72 h for the biomass to grow. About 25 g of fungal biomass was added to the flasks containing 100 ml of sterile distilled water and incubated for another 48h and filtered using whitmans filter paper no 1. The filtrate was then mixed with 100 ml of aqueous solution of 1mM concentration of silver nitrate (AgNO3) and kept in dark for 24 h at 29ºC was further used.

Minimum inhibitory concentration (MIC) of AgNPs against *E. faecalis* was determined by microbroth dilution technique as per clinical and laboratory standards guidelines.[12] Bacterial suspension of *E. faecalis* (ATCC 29212) strains grown on trypticase soy agar (TSA) plates at 35°C for 24 h were confirmed by Gram-Staining. Turbidity of the inoculum was adjusted to 1×10^8 CFU/ml.

10 µl of bacterial inoculum was added to culture medium containing 100 µl of series of dilutions of AgNPs in the wells of microtiter plates. The final concentration of AgNPs ranged from 80 to 1 µg/ml. One hundred microliters of culture medium with 10 µl of the bacterial inoculum without AgNPs was used as control group. Ten microliters Alamar blue (0.7 mg/ml) indicator was added to all the wells and incubated at 37°C for 24 h. After 24 h of incubation, the microplates were observed and the lowest drug concentration that inhibits bacterial growth is calculated as MIC. The color of each well was visually observed and the lowest concentration that does not result in color change from blue-to-pink color was recorded as MIC, and also, the optical density was measured using a microplate reader (Multiscan EX, Thermo Scientific, Vantaa, Finland) set at 600 nm. MIC was calculated as the minimum concentration of AgNPs that inhibited the bacterial growth as compared with control [Figure 1].

The antibacterial efficacy of biosynthesized AgNPs against *E. faecalis* in biofilm form was assessed using the dentin blocks according to Nascimento *et al*.[13] with slight modifications. The study was approved by Institutional ethical committee of HKES's Institute of Dental Sciences and Research, Kalaburagi.

Thirty dentin blocks were prepared from extracted human permanent maxillary anterior teeth. The crown and apical part of the root were sectioned using a rotary diamond disc to obtain the middle third of the root which was vertically sectioned into two halves and cementum was removed using the diamond bur to obtain the dentin blocks measuring 5 mm × 5 mm × 1 mm (width × length × thickness). The prepared dentin blocks were treated in an ultrasonic bath of 17% ethylenediaminetetraacetic acid (Prime Dental Products, Mumbai, India) for 5 min to remove the smear layer followed by 3% sodium hypochlorite (Prime Dental Products, Mumbai, India) for 5 min to remove the organic and inorganic debris and distilled water for another 5 min to remove all the chemicals used and sterilized by autoclaving. For evaluating antibacterial efficacy in biofilm form, all the dentin blocks were placed in 1 ml of TSA broth in individual test tubes and subjected to the second cycle of sterilization.

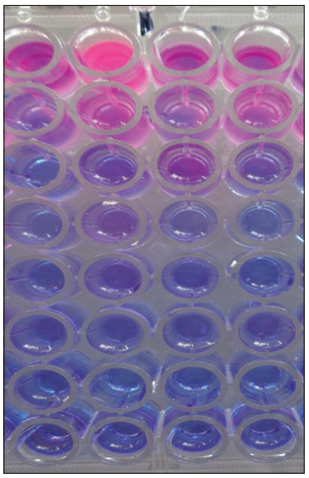

Figure 1: Microbroth dilution test plate of *Enterococcus faecalis* for minimum inhibitory concentration determination

Bacterial cell suspension prepared from overnight grown cultures at 37°C in TSA broth was centrifuged at 6000 rpm for 4 min after which the supernants were discarded, and collected cells were washed twice with sterile distilled water. The cells were suspended in 5 ml of TSA broth and incubated for 4 h at 37°C and turbidity adjusted to 0.5 McFarland standards (1×10^7 cells/ml) using a spectrophotometer.

For contamination of dentin blocks, 50 µl of the bacterial inoculum was transferred to presterilized individual test tubes containing 1 ml of the TSA broth and dentin block. Purity of the culture was checked by subculturing 5 µl from the incubated dentin blocks on TSA plates. The broth was replenished every alternate day, and the dentin blocks were contaminated for 2 weeks. After incubation, dentin blocks were washed twice with 5 ml of sterile distilled water and randomly divided into three groups (n = 10 each) and treated as follows: Group I: Sterile distilled water (control), Group II: AgNPs (MIC dose), Group III: 2% chlorhexidine gluconate (CHX) and incubated at 37°C for 24 h. At the end of 24 h, each dentin block was rinsed in saline and transferred to microcentrifuge tubes and shaken for 60 s (vortex AP 56). Serial decimal dilutions were prepared and 50 µl of the dilution was poured on TSA plates and incubated for 24 h followed by CFU/ml counting. The collected data were tabulated and subjected to statistical analysis.

Statistical analysis

The collected data was statistically analyzed by one-way ANOVA followed by post hoc Tukey honestly significant difference test at $P < 0.0001$ using the commercially available statistical software SPSS-20, (IBM Analytics, New York, United States of America).

Results

MIC of AgNPs for *E. faecalis* was determined as 30 µg/ml which resulted in complete inhibition of bacterial growth. For evaluation of antibacterial efficacy in biofilm mode, the mean colony count (CFU/ml (10^7)) for all the three groups was calculated [Table 1]. No significant difference was found between AgNPs and 2% CHX when compared to the control group with mean colony count being 2.4, 2.5, and 6.77 CFU/ml (10^7), respectively, at $P < 0.0001$ [Table 2]. The results of the study indicate biosynthesized AgNPs are as effective as 2% CHX against *E. faecalis*.

Discussion

Pulp and periapical diseases are biofilm-based infections, and elimination of bacteria in biofilm mode is the most difficult task encountered in endodontic therapy.[14] The root canal morphology, structure and composition of the biofilm, characteristics of microorganisms within biofilm, and limitations associated with the available disinfectants contribute to endodontic treatment failure.[15] Long-term subsequent exposure of bacteria to chemical disinfectants might induce resistance.[16] The bacteria in biofilm form are more resistant compared to the planktonic form. Therefore, destruction of the biofilm matrix and complete elimination of the bacteria are the difficult challenges encountered in root canal disinfection today.[17,18]

AgNPs exhibit potential antibacterial activity and does not allow to develop resistance.[19] Positively charged AgNPs interact with the negatively charged bacterial cell walls, adhere, and penetrate into the bacterial cell leading to the loss of cell wall integrity and permeability.[20] AgNPs exhibit potential antibacterial activity against both Gram-positive, Gram-negative bacteria including multidrug-resistant strains.[11,19,21] AgNPs have been recommended as root canal irrigating solution and intracanal medicament for root canal disinfection due to their effective antibacterial activity, biocompatibility, and ability to penetrate tissues and imparting antibacterial activity at smaller doses.[7] However, there are no studies indicating the application of biosynthesized AgNPs derived from fungi *F. semitectum* in root canal disinfection. Therefore, the present study highlights the application of fungal-derived biosynthesized AgNPs as antibacterial agents against 2-week-old *E. faecalis* biofilm.

E. faecalis is found in 90% of persistent infections after root canal therapy.[22] It is capable of forming biofilms and can penetrate the tissues. Moreover, it is resistant to most of the root canal disinfectants,[9] hence, chosen as test organism in the present study.

MIC is a quantitative approach to determine antibacterial sensitivity of test organism. It is the lowest concentration of a drug that inhibits the visible growth of microbes. MIC scores are often used by clinicians to select appropriate antimicrobial agent and to identify an effective dose for treatment. MIC determines the bacterial resistance to an antimicrobial agent. It is used as a research tool to determine the *in vitro* activity of new antimicrobial agents. *In vitro* determination of MIC of a drug against pathogens acts as a guideline

Table 1: Mean CFU/ml data collection for all samples

Sample (n)	Control (CFU/ml)	2% CHX (CFU/ml)	AgNPs (CFU/ml)
1	6.8	2.5	2.5
2	6.7	2.4	2.6
3	6.9	2.3	2.5
4	6.5	2.4	2.4
5	6.8	2.3	2.5
6	6.9	2.4	2.5
7	6.8	2.4	2.4
8	6.8	2.5	2.6
9	6.7	2.4	2.5
10	6.8	2.4	2.5

CHX: Chlorhexidine gluconate; AgNPs: Silver nanoparticles

Table 2: One-way ANOVA and *post hoc* Tukey honest significant difference multiple comparisons

	Control	2% CHX	AgNPs	Multiple comparison between	*P*
Mean	6.77	2.5	2.4	Control and 2% CHX	0.0010
SD	0.1160	0.0667	0.0667	Control and AgNPs	0.0010
P<0.00001				Control and AgNPs	0.0391

CHX: Chlorhexidine gluconate; AgNPs: Silver nanoparticles; SD: Standard deviation

for its *in vivo* application.[23] The MIC of AgNPs against *E. faecalis* in the present study was determined using microbroth dilution method, which is commonly employed technique and found to be 30 µg/ml. To evaluate the antibacterial efficacy against *E. faecalis*, the minimum dose (MIC dose) was standardized to evaluate the antibacterial activity for biofilm models.

In the present study, human extracted teeth were used to prepare the dentin blocks and to establish *E. faecalis* biofilm as they simulate the clinical conditions.[18] 2% CHX is used as positive control because it is a potent antimicrobial agent, has low-grade toxicity, adsorbs to dental tissues, exhibits substantivity and recommended as final root canal irrigant.[11,24]

The results of the present study showed that biosynthesized AgNPs have effective antibacterial efficacy against *E. faecalis* as chlorhexidine with mean CFU/ml (10^7) values of 2.4 and 2.5, respectively, which are in accordance to Wu D *et al*.[18] MIC of AgNPs derived from *Garcinia imberti* against *E. faecalis* was observed at a concentration of 62.5 mg/ml.[25] The results of the current study showed effective antibacterial activity against *E. faecalis* biofilm at 30 mg/ml. According to Ferraz *et al*., chlorhexidine is a potential root canal disinfectant.[26] Bo *et al*. reported 0.1% and 0.2% nanosilver gel is more effective on *E. faecalis* biofilm as compared to CHX and camphorated phenol.[27]

The antibacterial efficacy of biosynthesized AgNPs against *E. faecalis* in the present study indicates the application of biosynthesized AgNPs for endodontic disinfection as root canal irrigant and intracanal medicament. Despite the effective antibacterial efficacy of biosynthesized AgNPs against *E. faecalis* biofilm, the possible adverse effects such as cytotoxicity to periapical tissues, host cells, and root dentin staining should be considered for *in vivo* application. However, further *in vivo* and *in vitro* studies are required to optimize the effective use of biosynthesized AgNPs.

Conclusion

The present study highlights the effective application of biosynthesized AgNPs for endodontic disinfection creating a new horizon in endodontic therapy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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