RESEARCH PAPER

Effect of Ultrasonically activated Chitosan and Silver nanoparticle incorporated Chitosan on elimination of E. Faecalis biofilm - A Confocal Laser Scanning Microscopic in - vitro study

Krishna prasad L[1], Banashree Bhagwati[2], Gunjan chawla[3], Safiya Nikhat[4] *

[1]Professor & Hod department of conservative dentistry and endodontics, KVG dental college and hospital sullia Email: drkpdental@yahoo.co.in

[2]Post graduate student KVG dental college and hospital sullia, Email: Drbanashreeb@gmail.com

[3]Post graduate student KVG dental college and hospital sullia, Email: greenmango15@yahoo.com

*[*4]Post graduate student KVG dental college and hospital sullia, Email: safiyanikhat99@gmail.com*

ABSTRACT

Background: Complete elimination of microorganisms from the root canal system remains a major challenge in endodontics due to the persistence of biofilm-forming organisms such as Enterococcus faecalis. Chitosan and silver nanoparticles have shown promising antimicrobial properties, and their efficacy may be enhanced with ultrasonic activation.

Aim: To evaluate and compare the effect of ultrasonically activated chitosan and silver nanoparticle–incorporated chitosan on the elimination of E. faecalis biofilm using confocal laser scanning microscopy (CLSM).

Materials and Methods:

Sixty-three single-rooted premolars were prepared and inoculated with E. faecalis (ATCC 29212) for 15 days. Samples were divided into three groups (n=21): saline (control), 0.2% chitosan with ultrasonic activation, and silver nanoparticle–chitosan with ultrasonic activation. Passive ultrasonic irrigation was performed for 30 seconds. Biofilm viability was assessed using CLSM with BacLight staining to differentiate live and dead bacteria. Data was analyzed using ANOVA and post hoc Tukey tests ($p<0.05$).

Results: The saline group showed the highest live bacterial count and lowest bacterial count, indicating minimal antimicrobial activity. Ultrasonically activated chitosan significantly reduced live bacteria, while silver nanoparticle–chitosan demonstrated the highest dead bacterial count, indicating superior bactericidal action. Intergroup differences were statistically significant ($p<0.001$).

Conclusion: Ultrasonically activated chitosan is effective in reducing E. faecalis biofilm, while the addition of silver nanoparticles enhances bactericidal efficacy. This combination may serve as a promising adjunct in endodontic disinfection.

Keywords: Chitosan, Silver nanoparticles, Enterococcus faecalis, Ultrasonic activation, Biofilm, CLSM.

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INTRODUCTION

Primary endodontic infections are caused due to pulp tissue necrosis, colonized by microorganisms. The ultimate goal of endodontic treatment is to control microbial load in the complex root canal system. The smear layer is potentially infected, and its effective removal allows more efficient penetration of medications into the dentinal tubules, thereby providing a better interface between obturation materials and root

canal walls. (1,2,3,4) The success of endodontic treatment depends upon complete disinfection followed by debridement of the root canal system. Instrumentation alone cannot achieve complete elimination of bacteria

and debris in the root canals; thus, the role of effective irrigation is mandatory. (2,5)

Enterococcus faecalis (E. faecalis) is the predominant bacterium responsible for failure of root canal treatment and persistent infections. (6,7) It is a Gram-positive facultative anaerobe capable of withstanding extreme environmental conditions.(8) The prevalence of E. faecalis in primary endodontic infection is about 40% and in persistent or secondary infection ranges from 24% to 77%.(7,9) E. faecalis forms a calcified biofilm under harsh environmental conditions within the root canal system, leading to biofilm-mediated infections that are difficult to treat and contribute to increased antimicrobial resistance, ultimately resulting in secondary root canal infections.(7,10)

Chitosan is a naturally occurring cationic aminopolysaccharide copolymer composed of glucosamine and N-acetylglucosamine, obtained by partial deacetylation of chitin derived from crustacean shells.(11) Chitosan exhibits high chelating capacity along with properties such as biocompatibility, bioadhesion, biodegradability, and antimicrobial activity, making it an effective irrigant.(11) Antimicrobial agents capable of disrupting the extracellular polymeric matrix (EPM) can penetrate biofilms and eliminate bacteria. Silver nanoparticles (AgNPs) have gained attention due to their ability to penetrate tissues, interact with bacterial cells, and exhibit potent antimicrobial activity with good biocompatibility. (12)

The adherence of E. faecalis to root canal dentin using different irrigants such as sodium hypochlorite (NaOCl), saline, ethylenediaminetetraacetic acid (EDTA), and chlorhexidine (CHX) has been evaluated using confocal laser scanning microscopy (CLSM).(11) CLSM offers advantages such as visualization of live bacteria in dentinal tubules using vital staining, identification of labelled bacteria via in situ hybridization, and three-dimensional assessment of biofilm architecture within root canals.(12) Studies have also evaluated the depth of bacterial penetration into dentin and the effects of adjuncts like diode lasers using CLSM.(12)

Although instrumentation removes most contents from the main root canal, irrigation plays a crucial role in cleaning areas inaccessible to instruments. (13) Therefore, evaluating naturally occurring substances and nanoparticles for effective elimination of E. faecalis is essential for improving endodontic treatment outcomes.

AIM:

To assess and compare the effect of ultrasonic activated chitosan and silver nanoparticles mixed with chitosan on removal of Enterococcus faecalis biofilm adherent to root canal using confocal laser scanning microscope.

OBJECTIVES:

1) To assess the viability of E. faecalis in root canal by irrigating with 0.2% chitosan with ultrasonic activation using confocal laser scanning microscope.

2) To assess the viability of E. faecalis in root canal by irrigating with silver nanoparticles mixed with chitosan with ultrasonic activation using confocal laser scanning microscope.

3) To compare the effectiveness of ultrasonic activated Chitosan and Silver nanoparticles-chitosan mixture on removal of canal adherent E. faecalis biofilm.

Sample size estimation:

Using the formula,

$$n = \frac{2(SD)2(Z1-\alpha/2 + Z\beta)2}{(d)2}$$

where, SD = standard deviation- 1.5

$Z1-\alpha/2 = 1.96$ at 95% confidence interval

$Z\beta = 0.84$ AT 80% power

d = mean difference =0.91

Substituting the values, we get n = 20.6

10% sample is added to compensate for the sampling loss if any, thus the final sample was 21 per group

Methodology:

Ethical clearance was taken IECKVGDCH/FR04/2022-23

Sixty-three single-rooted premolar teeth were collected and maintained in phosphate-buffered saline (PBS) solution. The teeth were sterilized using a steam autoclave at 121°C for 20 minutes. The crowns were decoronated to obtain approximately 15 mm of uniform root length, and the working length was determined to be 1 mm short of the apical foramen (14 mm).

The root canals were prepared using ProTaper Gold rotary nickel–titanium instruments (Dentsply Maillefer) up to an apical size of 25 with a 0.06 taper. During instrumentation, 3% sodium hypochlorite and 17% EDTA were used as irrigants.

The tooth specimens were vertically sectioned along the mid-sagittal plane into two halves (mesial and distal). The split halves were re-approximated using utility wax placed over the root apex, and the specimens were embedded in a dental stone encasing.

Preparation And Inoculation of E. Faecalis Biofilm

A colony of Enterococcus faecalis (American Type Culture Collection [ATCC 29212]) was obtained. A suspension of E. faecalis was prepared in 2 mL of brain

heart infusion (BHI) broth, and the concentration was adjusted to 1 McFarland standard.

Each root canal was inoculated with 30µL of the bacterial suspension and incubated at 37°C for 15 days to allow biofilm formation. Following incubation, the canals were washed with 1 mL of phosphate-buffered saline to remove non-adherent bacteria from the canal walls. All microbiological procedures were performed under aseptic conditions.

Irrigation Procedure

The prepared teeth (n = 63) were randomly divided into three groups of 21 teeth each: Group 1 (n = 21): Saline (control group) – no activation, Group 2 (n = 21): 0.2% chitosan – ultrasonically activated & Group 3 (n = 21): Silver nanoparticle–chitosan mixture (1:1) – ultrasonically activated

ULTRASONIC ACTIVATION PROTOCOL

In Groups 2 and 3, passive ultrasonic activation of irrigants was performed using a stainless-steel ISO size 25 ultrasonic endodontic tip. The tip was activated inside the canal for 30 seconds using buccolingual and mesiodistal movements with a piezoelectric ultrasonic unit (Ultra X). The tip was maintained 1 mm short of the root apex.

Confocal Laser Scanning Microscopic Examination

The dentin specimens were placed onto microscope slides and stained using the BacLight viability stain. The samples were then examined under a confocal laser scanning microscope (CLSM), which was adjusted to detect fluorescein isothiocyanate and propidium iodide fluorescence. The BacLight stain consisted of two fluorescent dyes: fluorescein isothiocyanate (emission: 480/500 nm) and propidium iodide (emission: 490/635 nm). Two to three random areas of the biofilm on each dentin section were scanned using a step size of 2 mm.

Statistical Analysis

Data analysis was performed using SPSS version 21. Descriptive statistics were calculated and expressed appropriately. An independent t-test was used for intergroup comparison. A p-value of < 0.05 was considered statistically significant.

RESULTS

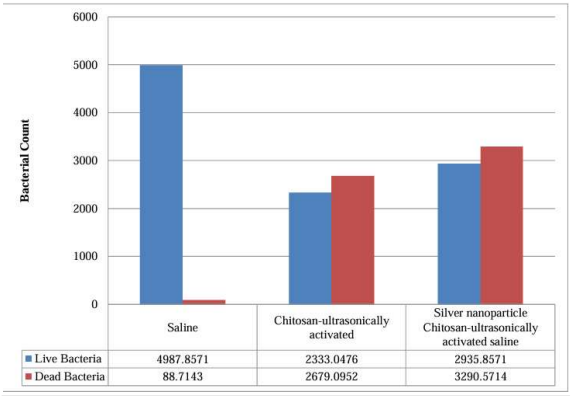
The saline group demonstrated the highest mean count of live bacteria (4987.86 ± 572.26), indicating minimal antimicrobial activity. In contrast, ultrasonically activated 0.2% chitosan showed a significant reduction in live bacterial counts (2333.05 ± 266.47). The silver nanoparticle–incorporated chitosan group also exhibited reduced live bacterial counts (2935.86 ± 481.38), although this reduction was less pronounced compared to plain chitosan. One-way ANOVA revealed a highly significant difference among the groups ($F = 193.638$, $p < 0.001$).

The saline group exhibited the lowest dead bacterial count (88.71 ± 9.77), indicating minimal bacterial killing. Ultrasonically activated chitosan showed a marked increase in dead bacterial count (2679.10 ±

Table No.1: Comparative Analysis of Live and Dead Bacteria Counts Following Treatment with Saline, Chitosan- Ultrasonically Activated, and Silver Nanoparticle Chitosan-Ultrasonically Activated Saline.
* Statistically significant difference among groups ($p < 0.001$) using appropriate inferential test (e.g., ANOVA).
SD – Standard deviation; SE – Standard error

		N	Mean	Std. Deviation	Std. Error	p value
Live Bacteria	Saline	21	4987.8571	572.25836	124.87701	**<0.001**
	Chitosan-ultrasonically activated	21	2333.0476	266.46603	58.14766	
	Silver nanoparticle Chitosan-ultrasonically activated saline	21	2935.8571	481.37618	105.04489	
	Total	63	3418.9206	1231.04214	155.09673	
Dead Bacteria	Saline	21	88.7143	9.76802	2.13156	**<0.001**
	Chitosan-ultrasonically activated	21	2679.0952	261.30823	57.02213	
	Silver nanoparticle Chitosan-ultrasonically activated saline	21	3290.5714	410.51511	89.58174	
	Total	63	2019.4603	1426.07663	179.66877	

261.31). The silver nanoparticle–incorporated chitosan group demonstrated the highest bactericidal effect, with the maximum dead bacterial count (3290.57 ± 410.52). ANOVA analysis revealed a highly significant difference among the groups ($F = 768.369$, $p < 0.001$). (table 1)



Graph: Comparative Analysis of Live and Dead Bacteria Counts Following Treatment with Saline, Chitosan-Ultrasonically Activated, and Silver Nanoparticle Chitosan-Ultrasonically Activated Saline.

Post-hoc Tukey HSD Analysis revealed, Live bacteria in both chitosan and silver nanoparticle–chitosan groups showed significantly fewer live bacteria compared to saline ($p < 0.001$). Plain chitosan was significantly more effective than silver nanoparticle–chitosan in reducing live bacteria (mean difference = 602.81, $p < 0.001$), whereas dead bacteria in both experimental groups showed significantly more dead bacteria compared to saline ($p < 0.001$). Silver nanoparticle–chitosan resulted in significantly more bacterial death than plain chitosan (mean difference = 611.48, $p < 0.001$). (table 2)

Overall, the saline group exhibited negligible antimicrobial activity, confirming its validity as a negative control. Ultrasonically activated chitosan demonstrated strong antibacterial efficacy, as evidenced by reduced live bacterial counts and increased dead bacterial counts.

Silver nanoparticle–incorporated chitosan, when ultrasonically activated, exhibited the highest bactericidal effect, particularly in terms of bacterial killing. However, plain chitosan showed a slightly greater reduction in live bacterial counts.

Table No.2: ANOVA Results for Live and Dead Bacteria Counts Between Different Treatment Groups.
* Statistically significant difference among groups ($p < 0.001$) using one-way analysis of variance (ANOVA).
df – Degrees of freedom

		Sum of Squares	df	Mean Square	F	Sig.
Live Bacteria	Between Groups	81354678.508	2	40677339.254	193.638	**<0.001**
	Within Groups	12604136.095	60	210068.935		
	Total	93958814.603	62			
Dead Bacteria	Between Groups	121351060.413	2	60675530.206	768.369	**<0.001**
	Within Groups	4738001.238	60	78966.687		
	Total	126089061.651	62			

DISCUSSION

Despite considerable advancements in endodontic disinfection, complete elimination of microorganisms from the root canal system remains a persistent challenge due to the complex anatomy and the resilient nature of biofilm-forming organisms such as Enterococcus faecalis. Conventional irrigants and intracanal medicaments, although effective to a certain extent, often fail to achieve complete bacterial eradication, particularly within dentinal tubules and inaccessible canal irregularities (3,4). Chitosan has emerged as a promising biopolymer with notable antimicrobial and chelating properties, while silver nanoparticles have demonstrated potent bactericidal effects through multiple mechanisms (1,14). Previous studies have evaluated these agents individually and, in some instances, in combination, reporting enhanced antimicrobial efficacy. (15) However, the existing literature predominantly focuses on their standalone application or incorporation into sealers and medicaments, with limited exploration of their use as

irrigant systems. (16) Furthermore, there is a paucity of data assessing the combined effect of chitosan and silver nanoparticles when used in conjunction with activation techniques such as passive ultrasonic irrigation, which is known to significantly enhance irrigant penetration and biofilm disruption (17,5). In addition, most studies have primarily reported overall bacterial reduction without distinctly differentiating between bacteriostatic and bactericidal outcomes, which are critical for a comprehensive understanding of antimicrobial efficacy. Therefore, there remains a clear need for studies that evaluate the synergistic potential of chitosan and silver nanoparticle–based irrigants under activated conditions, with simultaneous assessment of live and dead bacterial populations. The present study was designed to address this gap and provide a more detailed insight into the antimicrobial dynamics of such combination systems.

In the present study, the saline group showed the highest number of viable bacteria and the lowest number of dead bacteria, confirming its negligible antimicrobial effect. This was expected, since saline does not possess inherent antibacterial properties and mainly acts as a flushing agent. Similar findings have been reported previously, where saline failed to significantly reduce E. faecalis biofilms (1).

Ultrasonically activated chitosan, on the other hand, resulted in a marked reduction in live bacterial counts along with a notable increase in dead bacteria. Chitosan is known for its antimicrobial activity, which is largely attributed to its polycationic nature. This allows it to interact with negatively charged bacterial cell membranes, disrupting their integrity and increasing permeability, ultimately leading to cell death. Jaiswal et al. also reported comparable antibacterial efficacy of chitosan against E. faecalis (1). In addition, the use of ultrasonic activation likely contributed to the enhanced effect observed in this study, as it improves the penetration and distribution of irrigants within the root canal system. Previous studies have shown that passive ultrasonic irrigation promotes acoustic streaming and cavitation, thereby improving both smear layer removal and bacterial reduction (2,6).

Interestingly, chitosan alone demonstrated a greater reduction in live bacterial counts compared to the silver nanoparticle–incorporated chitosan group. This finding may be related to changes in the physicochemical properties of chitosan when combined with nanoparticles, which could influence its ability to penetrate dentinal tubules or interact effectively with the biofilm.

In contrast, the silver nanoparticle–chitosan combination showed the highest number of dead bacteria, indicating superior bactericidal activity. Silver nanoparticles are well known for their broad-spectrum antimicrobial effects. Their action involves disruption of the bacterial cell wall, production of reactive oxygen species, and interference with essential intracellular processes such as DNA replication. Halkai et al. also demonstrated strong antibiofilm activity of silver nanoparticles against E. faecalis, supporting the present findings (14). The enhanced effect observed with the combination may be due to a synergistic interaction, where chitosan facilitates membrane disruption while silver nanoparticles exert intracellular damage.

The difference observed between the reduction in live bacteria and the increase in dead bacterial counts suggests that these agents may differ in their mode of action. While chitosan appears to be more effective in inhibiting bacterial viability, the addition of silver nanoparticles seems to enhance the overall killing efficiency. This highlights the importance of evaluating both bacteriostatic and bactericidal effects when assessing antimicrobial efficacy.

The importance of combining mechanical and chemical methods for effective root canal disinfection has been well documented. Studies by Siqueira et al. and Byström and Sundqvist have shown that instrumentation alone is insufficient to completely eliminate microorganisms from the root canal system, emphasizing the need for effective irrigants (3,4). In recent years, adjunctive techniques such as ultrasonic activation have gained attention for their ability to improve disinfection outcomes. Plotino et al. highlighted that activation methods enhance irrigant penetration into complex canal anatomies, thereby improving their overall effectiveness (5).

The results of the present study support the growing evidence that activation techniques play a key role in maximizing the efficacy of irrigants. Ultrasonic activation not only improves irrigant distribution but also helps disrupt the biofilm structure, making bacteria more susceptible to antimicrobial agents.

Despite these encouraging findings, certain limitations should be acknowledged. This study was conducted under in vitro conditions, which may not fully reflect the clinical scenario. Factors such as host immune response and variations in canal morphology could influence the

outcomes in vivo. Additionally, although silver nanoparticles showed promising antibacterial effects, their long-term safety and biocompatibility need to be further evaluated before they can be routinely used in clinical practice.

CONCLUSION

Ultrasonically activated chitosan demonstrated effective antimicrobial activity against E. faecalis, while the addition of silver nanoparticles significantly enhanced bactericidal action. The combination showed superior bacterial killing compared to chitosan alone, whereas saline exhibited minimal effect.

Clinical Significance

The use of chitosan-based irrigants, particularly when combined with silver nanoparticles and ultrasonic activation, may improve root canal disinfection by enhancing bacterial elimination. This approach has the potential to serve as an effective adjunct to conventional irrigation protocols, especially in cases with persistent infections.

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