ORIGINAL ARTICLE

Antibacterial Efficacy of Silver Nanoparticles Impregnated Calcium Hydroxide: An in Vitro Study

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OBJECTIVE: To compare the in-vitro antibacterial effectiveness in terms of enterococcus faecalis elimination of conventional unmodified calcium hydroxide paste with an experimental calcium hydroxide impregnated with 1% silver nanoparticles.
METHODOLOGY: This in vitro study was conducted during the period July 2019 - January 2020 at Materials Research Laboratory, Centralized Resource Laboratory (University of Peshawar) and Pathology Laboratory (Peshawar Medical College). Sixty human premolar teeth having single root with mature apices were obtained. These were distributed into two groups randomly (n=30); experimental group for testing the antibacterial efficacy of calcium hydroxide impregnated with 0.1% by weight silver nanoparticles and the other was used as a control group for testing the antibacterial efficacy of unmodified calcium hydroxide. Dentine specimens (size 4 x 4x 1mm) were prepared from the teeth using slow speed cutting saw machine. Smear layer was removed with EDTA. Specimens were autoclaved to sterilize them. Then each specimen was contaminated with enterococcus faecalis and incubated anaerobically for 24 hours at 37?C. After the application of medicaments, the zone of inhibition was measured and number of viable bacteria were determined using SEM. Statistical significance was calculated using t-test. $p<0.05$ was taken as significant.
RESULTS: After the application of medicament, the zone of inhibition was greater (9.63mm) in experimental group when compared to control group (4mm) ($p<0.001$). Number of viable bacteria before application of medicament were 239 and 208 while after application of medicament reduced to 90 and 54 in control and experimental groups respectively ($p<0.001$).
CONCLUSION: The addition of silver nanoparticles to calcium hydroxide significantly enhanced its potential to eliminate biofilm of enterococcus faecalis on dentin specimens.

KEYWORDS: Silver nanoparticles, Calcium hydroxide, Root canal infections, Root canal medicament.
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INTRODUCTION

The success of endodontic treatment depends on several factors in which the reduction or elimination of infections is one of the most important aspect. In teeth with apical periodontitis, microorganisms can persist after the endodontic treatment and remain harbored in the dentinal tubules.[1] The mechanical preparation used during endodontic treatments alone do not completely eliminate the infection and inflammatory processes in the periapical tissues. Thus, the application of an intra canal medicine helps in eradication of microbes that exists even after preparation of the canal, therefore creating an atmosphere for repair of periapical tissue.[2]

Enterococcus faecalis is anaerobic gram-positive cocci accountable for majority of endodontic treatment failures.[3] It has the ability to withstand adverse environmental conditions.[4] It can invade into the tubules inside dentine and has the ability to form biofilm.[5] To eliminate such

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microorganisms, antimicrobial intra canal medicament is recommended.[6] Among the intra canal medicaments, calcium hydroxide is most frequently used because of its wide antimicrobial spectrum.

Calcium hydroxide $Ca(OH)_2$ is commonly employed as an intra canal medicament. It releases hydroxyl ions which causes high alkalinity. Nevertheless, the ability of calcium hydroxide in elimination of bacteria from the root canal has been questioned. Antibacterial ability of calcium hydroxide in aqueous environment is linked to the discharge of hydroxyl ions. It can cause damage to the cytoplasmic membrane, bacterial DNA as well as can cause protein denaturation.[7] However, calcium hydroxide is not as much effective when used for canal disinfection having established enterococcus faecalis biofilm.[8]

Recently, numerous studies have introduced silver nanoparticles as an antimicrobial agent.[9] Silver nanoparticles have antimicrobial activity and are biocompatible[10], silver ions can cause damage to the bacterial cell wall. These are productive against many microorganisms including E. faecalis.[11] Nanoparticles have /polyanionic polycationic properties with a high surface area and positive charge density, which increases their antibacterial activity.[12] The particle size was also related to antimicrobial activity; the smaller particles give more bactericidal effects compared to larger particles.[13]

Calcium hydroxide do not always eradicate enterococcus faecalis biofilm from root canals.[14] Hence, it is essential to introduce advanced endodontic intracanal medicament approaches that are successful in eradicating biofilm bacteria inside the root canals. Therefore, the aim of this was to determine the antibacterial efficacy of calcium hydroxide cement containing silver nanoparticles.

METHODOLOGY

The research protocol and the publication of results were approved by the institutional review board (Prime/IRB-2019-313). The experimental work was performed at the Department of Dental Materials, Peshawar Dental College, Pathology Laboratory of Peshawar Medical College and Material Research and Centralized Research Laboratory of Peshawar University.

Total of sixty test specimens were prepared. Thirty specimens (n=30) were made to test the antibacterial ability of commercially available calcium hydroxide paste (Calcipex,Nishika Japan) while the remaining thirty specimens were used to test the antibacterial activity of calcium hydroxide paste containing 1% by weight of silver nanoparticles (Alfa aeser, Germany). Experimental silver nanoparticles impregnated calcium hydroxide was prepared by mixing 1 mg of silver nanoparticles with 1 gm of calcium hydroxide. Sixty human premolars having single root and mature apices were obtained to prepare dentine specimens.

Teeth were cleaned from gross debris by using ultra sonic scaler and then stored in normal saline. After cutting off crown and apical portion, teeth were sectioned vertically along midsagittal plane into equal halves. The cementum from root surface was removed by using diamond bur. Dentin sections were made having dimensions of 4 x 4x 1mm (width x length x height). Smear layer from the specimens was removed by placing it in EDTA solution for four minutes. All the specimens were washed with sterile water for one minute and placed in autoclave (Hirayama, Japan) for 15mins at 121°C. Brain heart infusion was used to incubate the specimens at 37°C for 24 hrs.

Enterococcus faecalis used in this study was obtained from pathology laboratory of Lady Reading Hospital, Peshawar Pakistan. The bacteria were plated on BHI broth containing 1.5% (wt /vol) agar and incubated anaerobically at 37°C for 24 hrs to obtain colonies. Gram staining was performed and the grown colonies of bacteria were placed in sterile BHI broth at 37°C. Sterilized specimens of dentin were placed in test tubes having E.faecalis for bacterial inoculation on dentine specimen. Fresh BHI broth was exchanged every second day to eliminate dead cells and to confirm viability of bacteria. The specimens were removed from tubes after incubation and rinsed with sterile phosphate buffered saline to remove the culture medium and non adherend bacteria. The zone of inhibition of E.Faecalis was measured and number of viable bacteria were observed under SEM.

One pellet (0.01g) each of the control and experimental groups were placed on infected dentine specimens on a sensitivity discs. Then anaerobic incubation of these specimens was conducted at 37°C for 14 days in a 100% humid environment. Then each specimen was washed with saline to eliminate the tested medicament. Citric acid (0.5%) was used to neutralize the treated specimens treated with calcium hydroxide. Then the specimens were examined for zone of inhibition and number of viable bacteria was determined by using SEM.

Data analysis was performed using the statistical package for social sciences (SPSS) version 22. Mean values and standard deviations of the number of viable bacteria on dentine specimens were computed. Student's t test was used for estimating the significance of the differences of the mean values. $p < 0.05$ was used for statistical significant.

RESULTS

Mean and standard deviation of zone of bacterial colonies,

zone of inhibition of bacterial colonies and number of viable bacteria in both control and experimental groups before and after application of medicament are given in Table 1. Zone of bacterial colonies before application of medicament shows that both groups (control and experimental) contain similar number of colonies with mean value of 20 mm. After the application of medicament, the zone of inhibition of bacterial colonies was more in experimental group (9.63±2.09) than control group (4±2.86) showing statistically significant difference ($p<0.001$). Number of viable bacteria before application of medicament were 239± 60.3 and 208.03±65.22 while after application of medicament reduced to 90±37.96 and 54.13±23.3 in control and experimental groups respectively ($p< 0.001$) (Table 1).

Table 1: Zones of bacterial colonies, zone of inhibition of bacterial colonies and number of viable bacteria observed for control and experimental groups

	Groups	Mean (mm)	Std.Dev	Mean difference	P-value
Zone of bacterial colonies before application of medicament	Control	20.0	0.00	16±2.87	<0.001
Zone of inhibition of bacterial colonies after application of medicament	Control	4.00	±2.87		
Zone of bacterial colonies before application of medicament	Experimental	20.00	0.00	153.9±81.2	<0.001
Zone of inhibition of bacterial colonies after application of medicament	Experimental	9.63	±2.09		
No. of viable bacteria before and after application of medicament in the control group	Control (before)	239.00	±60.33	10.3±2.1	<0.001
	Control (after)	90.0	±65.32		
No. of viable bacteria before and after application of medicament in the experimental group	Experimental (before)	208.03	±37.96	149±54.33	<0.001
	Experimental (after)	54.13	±23.20		

DISCUSSION

The current study was conducted to compare the in vitro antibacterial effectiveness of two root canal medicaments in terms of elimination of bacterial colonies and reduction in the number of viable E. faecalis during a test period of two weeks. The null hypothesis was rejected since experimental calcium hydroxide having 1% silver nanoparticles statistically significantly reduced the E. faecalis number as compared to the plain calcium hydroxide. In this study E. faecalis was used for the evaluation of antibacterial efficacy of root canal medicaments, as it has a key role in the aetiology of constant root canal infections. E. faecalis is responsible for high rate of root canal failures because these bacteria can stay alive in adverse environment.[15] In our study, permanent teeth were used to replicate the real clinical situation to the possible extent.

Samiei et al reported better efficacy of calcium hydroxide containing silver nanoparticles when compared to conventional calcium hydroxide in the treatment of root canal infections.[16] Balto et al (2020) treated dentine specimens with $Ca(OH)_2$ containing 0.02% AgNPs. The results of their study showed that the mixture of $Ca(OH)_2$ and AgNPs exhibited marked antibacterial activity against 3-week-old E. faecalis biofilms. It has been observed that silver has a high affinity for negatively charged molecules within bacterial cells, disables critical functions of bacteria and consequently prevents bacterial proliferation and formation of biofilm.[17] The inhibitory effect of the mixture of $Ca(OH)_2$ and AgNPs on E. faecalis biofilms in our study is similar to that reported in numerous studies regardless of the variation in the assessment approach.[16,17]

Contrary to our findings, Yousefshahi et al reported statistically insignificant results when E. faecalis was treated with 1% AgNPs impregnated calcium hydroxide for 24 hours as compared to conventional calcium hydroxide.[18] The difference in the results of the two studies might be due to variation in shape and size of the silver nanoparticles.

Javidi et al reported that after one week of incubation, the number of colony forming units of E. faecalis were considerably less in specimens treated with calcium hydroxide containing silver nanoparticles as compared to unmodified calcium hydroxide paste ($p<0.001$.[19] Similarly, Afkhami et al reported that the efficacy of calcium hydroxide containing silver nanoparticles against E. faecalis was better than unmodified calcium hydroxide paste when incubated for one week and one month.[20] The findings of both the studies are in agreement with the findings of our study.

One of the limitations of this study was that only one concentration of AgNPs in combination with calcium hydroxide and one time period of two weeks was assessed and hence the maximum concentration of AgNPs which could be added to calcium hydroxide and the minimum time period showing the maximum efficacy was not ascertained.

Although E. faecalis is the commonest bacterial strain present in most patients of persistent intra-radicular infections, but it also needs to understand that the infected root canal system typically has more than one species of bacteria. So the intra canal medicaments showing efficacy against E. faecalis in in-vitro research may not be essentially work well in poly-microbial infection in vivo studies of endodontic origin. However, our investigation may serve as a baseline for further research in this area that could assess the influence of silver nanoparticles and its various combinations on other endodontic pathologies.

CONCLUSION

Within the limitations of this study, it could be concluded that calcium hydroxide containing silver nanoparticles might

be more effective in elimination of bacteria from root canal system than conventional unmodified calcium hydroxide. Further studies are required to explore its efficacy in in-vivo along with possible cytotoxicity and other adverse effects like tooth discolouration etc.

CONFLICT OF INTEREST

None declared

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