Original Article

Enhancing the antibacterial activity of the gold standard intracanal medicament with incorporation of silver zeolite: An *in vitro* study

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Abstract

Background: *Enterococcus faecalis* is a persistent organism that plays a major role in the etiology of persistent periradicular lesions after root canal treatment has been associated with different forms of periradicular disease including primary endodontic infections and persistent infections. The present study compares the antibacterial activities of calcium hydroxide, calcium hydroxide mixed with silver zeolite, and calcium hydroxide mixed with 2% chlorhexidine against *E. faecalis* using direct contact test. **Materials and Methods:** The test materials of the *in vitro* experimental study were grouped as group 1—calcium hydroxide mixed with sterile water, group 2—2% silver zeolite added in calcium hydroxide mixed with sterile water, and group 3—calcium hydroxide mixed with 2% chlorhexidine. The bottom of microtiter plate were coated with freshly mixed tested material and a 10 µL of bacterial suspension was placed. After 1 h of incubation at 37°C, brain–heart infusion (BHI) broth (245 µL) was added and mixed for 2 min. These were designated as "subgroup 1" wells. A volume of 15 µL of broth then transferred from subgroup 1 wells to an adjacent set of four wells containing fresh BHI medium (215 µL); these wells were designated as "subgroup 2"' wells. The optical density was measured by a spectrophotometer after the first day, third day, and seventh day. One-way analysis of variance (ANOVA) and Tukey tests were performed for the analysis. **Results:** Calcium hydroxide mixed with silver zeolite showed maximum antibacterial activity. **Conclusion:** Silver zeolite can be added in calcium hydroxide to enhance the latter's antibacterial activity against *E. faecalis*.

Key words: *Calcium hydroxide, Enterococcus faecalis, zeolite*

INTRODUCTION

Microorganisms and their by-products are the foremost sources of pathosis in pulp and the periradicular tissues. The polymicrobial flora in endodontics is dominated by obligate anaerobes. The microorganism most commonly isolated from persistent lesions is *Enterococcus Faecalis* ranging from 24% to 77%.[1] In endodontics,

Access this article online	
Quick Response Code:	Website: www.jispcd.org
	DOI: 10.4103/2231-0762.175418

How to cite this article: Ghatole K, Gowdra RH, Azher S, Sabharwal S, Singh VT, Sundararajan BV. Enhancing the antibacterial activity of the gold standard intracanal medicament with incorporation of silver zeolite: An *in vitro* study. J Int Soc Prevent Communit Dent 2016;6:75-9.

successful reduction or complete elimination of the contagion will be a crucial factor for the prediction of success or failure of root canal treatment. The most common methods employed to eliminate the infectious matter from the canals are by cleaning and shaping the root canal using various mechanical and rotary endodontic instruments and irrigants. Due to the complexity of root canal system, in spite of thorough chemomechanical preparation a few of the bacteria may remain in the ramifications, isthmuses, apical deltas, and dentinal tubules often resulting in failure of root canal treatment.[2]

The most fundamental therapy that is an important adjunct to root canal therapy is intracanal medicament, which is considered an important mainstay that helps reduce the microbial flora beyond the levels previously achieved at the time of canal preparation. Intracanal medicaments are particularly useful in those areas which are not negotiated by instruments or irrigants, as they penetrate into these areas. These intracanal medicaments have lasting effect; between the appointments, they ensure reduction in the risk of proliferation and reorganization of the residual bacteria that can cause reinfection of the root canals. The additional benefits of the intracanal medicaments are as follows: They provide relief from pain, exudate control in weeping canals, and boost apical healing.[3]

The gold standard material in the practice of endodontics is calcium hydroxide that on a stand-alone basis performs most of the requisite functions and is considered as an ideal intracanal medicament. When calcium hydroxide is dissolved in water, it dissociates and produces hydroxyl and calcium ions. The antimicrobial uniqueness of hydroxyl ions is that it alkalinizes the mixture. The destruction of bacterial cell wall and protein structure is attributed to its high pH.[3] Conversely, all endodontic infections are not resolved effectively and equally by calcium hydroxide.[3] Hence, other medicaments have been added in an attempt to improve its antibacterial activity. Metallic silver is one of the common elements tried in dentistry for its known antibacterial properties. Zeolites are aluminum silicate crystalline structures that present void spaces that hold cations such as silver and zinc.[4] Silver-containing zeolite has been added to various dental materials, such as glass ionomer cement, mineral trioxide aggregate (MTA), and resin, that have been shown to enhance the antibacterial properties of these dental materials.[5-7]

Many other materials have been added to dental materials to increase their antibacterial properties. Chlorhexidine being one of them, it consists of cationic molecules that bind to the bacterial cell walls that are negatively charged, alter the cell's osmotic equilibrium, and finally kill the bacteria. It also possesses a unique property called substantivity (residual antimicrobial effect).[8] In endodontics, it has dual usefulness, i.e., as an irrigants and as an intracanal medicament. It is beyond any doubts that chlorhexidine inhibits the growth of various species of bacteria commonly prevailing in endodontic infections. It has bacteriostatic activity at low concentrations. The bactericidal action at higher concentrations of chlorhexidine is attributed to the fact that it causes coagulation and precipitation of cytoplasm.[9]

Direct contact test methodology was adopted as given by Weiss *et al*. (1996). It relies on two things—first is direct and second is close contact between the test organism and the test material in question regardless of the diffusibility and solubility of the antimicrobial components. It allows water-insoluble materials to be tested under various test conditions such as aging. Direct contact test is a qualitative and reproducible method with better control of the confounding factors that shows indifference to the size of inoculum that is brought in contact with the test material.[10] It is based on the readings of the transmittance values in the spectrophotometer, which provides turbidimetric determination of microbial growth.

The purpose of the present study was to compare the antibacterial activities of calcium hydroxide, calcium hydroxide mixed with silver zeolite, and calcium hydroxide mixed with 2% chlorhexidine against *E. faecalis* using direct contact test.

MATERIALS AND METHODS

Test microorganism

Facultative strain of *E. faecalis* (ATCC 29212) was grown on a brain–heart infusion (BHI) agar plate (with 5% defibrinated sheep blood). Microorganisms were subcultured on nutrient agar medium to confirm their purity. Facultative strain of *E. faecalis* was inoculated individually into tube containing 5 mL of sterile 85% saline. The suspension was adjusted spectrophotometrically at 800 nm to match the transmittance of 90T (equivalent to 0.5 McFarland scale = 1.5×10^8 C.F.U).

Test materials

- Group 1: Calcium hydroxide powder was dispensed and mixed with sterile distilled water in a ratio of 1:1 as the control group (negative control).
- Group 2: Silver zeolite (Sigma Aldrich Chemie GmbH, Germany, Batch #06306CJ) was added to 2% mass fraction of calcium hydroxide powder and mixed with sterile distilled water.
- Group 3: Calcium hydroxide was mixed with 2% chlorhexidine (Vishal Dentocare, Ahmedabad, Gujarat) in a ratio of 1:1 (positive control).

Direct contact test

Direct contact test is based on determining the turbidity of bacterial growth in 96 well microtiter plates. In the present study, all the test materials were mixed freshly according to the manufacturer's instructions. The bottom and the side walls of four wells were coated to a height of 2 mm with each of the mixed test material using a cavity linear applicator. A 10 μL bacterial suspension was placed on the test material. After the liquid evaporated ensuring a direct contact between the bacteria and surfaces of the test material, 245 μL of BHI broth was added, mixed for 2 min. A volume of 15 μL of the ensuring bacterial suspension was transferred into an adjoining set of four wells having fresh medium (215 μL) that was again mixed for 2 min. The kinetics of bacterial outgrowth in each well was then followed by continuous measurements by enzyme-linked immunosorbent assay (ELISA) reader measured at 630 nm. Densitometric readings were taken on the first, third, and seventh day with each set of samples.

One-way analysis of variance (ANOVA) was used to compare the mean optical density using Statistical Package for the Social Sciences (SPSS) version 18.0 software (SPSS-Inc., Chicago, IL). *Post hoc* analysis for multiple comparisons was performed using Tukey test. The level of significance was set at 0.05.

RESULTS

The results of the antibacterial effects of test materials from direct contact test are presented in Table 1. On day 1, calcium hydroxide mixed with silver zeolite showed maximum antibacterial activity followed by calcium hydroxide mixed with 2% chlorhexidine. The least antibacterial activity was seen with calcium hydroxide mixed with sterile distilled water. Similar results were seen at set material at 3-day and 7-day interval.

Table 1: Mean optical density readings on the first day, second day, and seventh day

Day	Time (h)	Mean optical dentistry			*P* value
		Group 1	Group 2	Group 3	
First day	1	0.35	0.27	0.31	0.00*
	7	0.34	0.24	0.28	0.00*
	12	0.32	0.22	0.26	0.00*
Third day	1	0.21	0.16	0.18	0.00*
	7	0.19	0.15	0.17	0.50*
	12	0.15	0.13	0.14	0.29
Seventh day	1	0.17	0.09	0.12	0.00*
	7	0.11	0.09	0.08	0.184
	12	0.07	0.04	0.06	0.12

**Statistically significant, one-way ANOVA, and P value set at 0.05*

DISCUSSION

Complete elimination of the bacteria from the pulp space leads to the success of the endodontic treatment. The number of bacteria in an infected root canal is usually in the range of 10^2–10^8.[1] *E. faecalis* is the most prevalent organism cultured from nonhealing endodontic cases. It has the capacity to endure prolonged periods of starvation and can form biofilms showing resistance to phagocytosis, antibodies, and antimicrobials.[11] Heling *et al*. showed that *E. faecalis* can withstand the ecologically amenable conditions making it challenging to eliminate them.[12] Peciuliene *et al*. determined presence of *Enterococcus species* in filled root canals with chronic apical periodontitis.[13] Sedgley *et al*. isolated 89.6% of *E. faeaclis* using quantitative real-time polymerase chain reaction in retreatment cases.[14] Thus, the elimination of the *E. faecalis* from the root canal is essential.

Clinical disinfection is done with a combination of intracanal irrigants and medicaments along with routine chemomechanical preparation. Calcium hydroxide has been used as the test material in the present study, as it the most popular and commonly used intracanal medication considered as the gold standard. However, Baker *et al*. reported that calcium hydroxide had poor antibacterial activity against *E. faecalis*.[15] Calcium hydroxide has good antimicrobial activity that is related to the production and release of hydroxyl ions in an aqueous setting. The extreme reactivity of hydroxyl ions can be ascribed to its highly oxidant free radicals that rarely diffuse away from the sites of generation.[3] A number of products have been incorporated to increase the antibacterial property of calcium hydroxide.

The inclusion of chlorhexidine in the present study is justified by the fact that it has demonstrated antimicrobial properties when used as an intracanal

medicament. Chlorhexidine and calcium hydroxide combination has demonstrated better antibacterial activity than calcium hydroxide mixed with sterile water at 1, 3 and 7 days. Similar results have been demonstrated previously by Ballal, Kundabala, Zarella.[16,17] This could be because the combination produces more reactive oxygen species with satisfactory physical and chemical properties that increase the antimicrobial action of calcium hydroxide.

Calcium hydroxide mixed with silver zeolite showed the maximum antibacterial activity against *E. faecalis* at 1, 3 and 7 days. Metallic silver as its constituent has highest antibacterial activity among metal ions.[4] Zeolite is a porous crystalline material of hydrated sodium, aluminosilicate, which exhibits a strong affinity for silver. Zeolites have void spaces within frameworks, 3–10 Å in a diameter, that are capable of hosting cations such as silver or zinc. Zeolite electrostatically binds to silver resulting in gradual, stable, and long-lasting release of silver ions from zeolite. The antibacterial activity could be due to silver ions that bind to zeolite and lead to a stable, gradually diffusing, and long-lasting release of silver ions.[18,19] Kawahara *et al*. suggested silver zeolite as a useful vehicle to enhance the antibacterial activity of the dental materials.[20] Davies *et al*. showed that antimicrobial activity of glass ionomer cement could be enhanced by incorporating silver zeolite with it. Adding 2% silver zeolite resulted in a significant increase in the silver release and the antibacterial effect was not promoted by the higher ratio of Ag–Zn–Zeolite in Hotts *et al*.[21] study while Cinar *et al*.[22] demonstrated an increased antimicrobial affect proportional to its concentration. Hence, 2% silver zeolite was used.

Direct contact test was used to assess the antibacterial activity as this test is virtually independent of the diffusion of the test material; the results showed unresponsiveness to the size of the inoculums and standardized measurements both in the presence and absence of the tested material.[10]

The results of the present study should be viewed in the light of limitation since root canal system harbor and species of microorganism and *in vitro* antibacterial activity of silver zeolite may be challenged in the complex environment of the oral cavity. Hence, additional studies regarding the use of same medicament *in vivo* should be conducted. The interaction of intracanal medicaments with microorganisms is a very complex mechanism and is still unknown. Further studies are needed to check the effect of silver zeolite on physical properties of calcium hydroxide, e.g., setting and working time. In an *in vitro* study, the addition of zeolite to root filling material did not increase the cytotoxic and hemolytic activity.[5] The teratogenicity of nanosilver in humans is unknown because no cases or studies have been reported in the literature. Thus, the nanosilver assessment in humans for potential teratogenic effects is imperative.[23]

CONCLUSION

Within the limitations of this study, mixing silver zeolite with calcium hydroxide enhances the latter's antibacterial activity against *E. faecalis.*

Financial support and sponsorship

The present study did not receive funds or grants from any of the agencies in India or Abroad.

Conflicts of interest

There are no conflicts of interest among the authors.

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