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Comparative Evaluation of Antimicrobial Efficacy Of Calcium Hydroxide, Propolis, Calcium Hydroxide Combined With Chitosan And Chitosan Against Enterococcus Faecalis: An In Vitro Study.

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Abstract

Aim: The purpose of this study is to investigate and compare the effectiveness of calcium hydroxide, propolis, calcium hydroxide combined with chitosan and chitosan for the elimination of E. faecalis.

Method: Four intracanal medicaments calcium hydroxide, propolis, calcium hydroxide combined with chitosan and chitosan were used against E faecalis. The strain were inoculated in blood agar and incubated at 37^0C for 24hrs. For the agar diffusion test, petri dishes with 20 ml of brain heart infusion broth were inoculated in 0.1 ml of microbial suspensions, using sterile swabs that would be placed on the medium, obtaining growth injunction. On the surface of agar plates, wells of 5 mm in diameter and of 18 µL in capacity were formed by using sterile gel borer. The samples of concentration 100 µg/mL were loaded by using a sterile micropipette. The 15 µL of suspension was placed in each well and was incubated at 37 ^{0}C ± 2 ^{0}C for 24 hours. One way ANOVA followed by

Tukey's post-Hoc test will be used. P value <0.05 will be considered in significant.

Result: Calcium hydroxide combined with chitosan and propolis showed better results against E faecalis.

Conclusion: Calcium hydroxide combined with chitosan showed better results against E faecalis. It appears that both has synergistic action and can be used as an intracanal medicament.

Keywords: E faecalis, chitosan, intracanal medicament, antimicrobial efficacy.

Introduction

Elimination of microorganisms and complete removal of pulp tissue from the root canal system is of dominant

magnitude during endodontic therapy. The root canal success mainly depends on mechanical preparation, irrigation, microbial control, and complete filling of the root canal system. Microorganisms, bacteria, and their products are considered as the etiological agents of pulp necrosis and periradicular lesions. They may survive during endodontic procedures due to anatomical structural complexities and limitations of access by instrumentation and irrigants. To ensure complete elimination of root canal bacteria, effective antimicrobial agents are required for a predetermined time period for predictable eradication of remaining bacteria.(1).

Organism commonly found in cases of failed endodontic infections and endodontic flare-ups is *Enterococcus faecalis*.It has the ability to survive in root canal system as a single organism without the support of other bacteria and is small enough to proficiently invade and live within the dentinal tubules.(2) Many studies have reported that *E. faecalis* are able to invade dentinal tubules to variable depth. So,to ensure complete elimination of root canal bacteria, an effective antimicrobial agent in the root canal is required for a predetermined time period for complete eradication of any remaining bacteria.

Calcium hydroxide has been widely used as an intracanal medicament in endodontics, and it has been demonstrated that *E. faecalis* have been reported to be resistant to the antimicrobial effect of calcium hydroxide (3).

Natural products have been used in dental and medicinal practises for thousands of years and have become more popular today. Propolis is a naturally occurring resinous substance that honey bee collect from various plants and mix it with wax flakes and their saliva in the hive. This mixture is what they use to cover hive walls, fill cracks or gaps and embalm dead invaders [7]. The chemical composition of propolis is very complex.The chemical composition of propolis varies widely due to climate, season and location. Also, the chemical formula is not stable [8].Propolis has various potential uses in oral health [9-11]. It has been shown to possess antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant, antitumour and immunomodulatory effects. Among these functional properties, antibacterial activity has been linked mainly to flavonoid content.

To increase the intracanal medicament stability, insolubility chitosan can be used as a drug carrier where it has added advantage of slow and controlled release of intracanal medicament.[4,7,8].Chitosan is produced by the partial deacetalylation of chitin. Chitin is the second most abundant natural polysaccharide composed of β (1→4) linked N-acetyl glucosamine units. Chitosan has a number of important pharmaceutical applications. It has been used in drug delivery, has an absorption enhancer, colon targeting and gene delivery.[9] Till date there is no sufficient data on the antimicrobial effect of intracanal medicaments using propolis and chitosan as a carrier on *E. faecalis.*

Materials and Methods

Preparation of Media and Culture Plate : A 5.8 gm of agar was mixed with 100 mL of distilled water in a mixing jar and agitated in circular motion. After thorough mixing of the ingredients it was sterilized in the autoclave for 15 minutes at 15 lbs and 121° C. The contents were poured into the sterile culture plates to a height of 5 mm and allowed to cool at room temperature .The culture media began to solidify below 58-60°C and got completely solidified in 30 minutes. The plates were then placed in an incubator for removal of moisture.

Preparation of Brain Heart Infusion (BHI) Broth for Enterococcus Faecalis

A 20 mL of BHI broth was taken in a test tube and heated in the Bunsen burner for 60 seconds and allowed to cool at room temperature .*Enterococcus faecalis* (ATCC 29212)

strain was taken in a loop and mixed in the prepared BHI broth by shaking the loop into the broth followed by shaking the test tube in circular motion .The test tube was then kept in the incubator for four hours before inoculation.

Preparation of Sample

Figure 1

The antibacterial activities of chitosan ,calcium hydroxide ,propolis and combination of chitosan and calcium hydroxide were tested on E faecalis by well diffusion method .The organism was incubated under aerobic condition. The agar plate was prepared in sterile glass petri dishes and kept overnight for sterility at 37°C.

On the surface of agar plates, wells of 5 mm in diameter and of 18 µL in capacity were formed by using sterile gel borer. The samples of concentration 100 µg/mL were loaded by using a sterile micropipette.The 15 µL of suspension was placed in each well and was incubated at 37 ^{0}C ± 2 ^{0}C for 24 hours.

Measurement of Zone Of Inhibition

After 24 hours incubation at 37°C, the inoculated agar culture plates were analysed for zone of inhibition. For measuring the diameter of zone of inhibition for each culture plate, the following method was used. By using the divider and ruler, the shortest diameter of the inhibition zone was measured as D1 and the longest diameter was measured as D2 and the average of the two was recorded as the “Diameter of zone of bacterial inhibition” for that culture plates.Following 24 hours incubation at 37°C, the culture plates were examined in a well-lit area for zone of bacterial inhibition. The zone of inhibition was seen as a round to oval clear area around the central well devoid of any bacterial growth.

Statistical analysis

Data collected by experiments were computerized and analyzed using the statistical package for social sciences (SPSS version 16.0).

Since the data were of continuous type ,parametric test were used for analysis.Mean (x) and standard deviation (SD) were calculated.One way analysis of variance (ANOVA) test was used for multiple group comparisons followed by Tukey post-hoc for group wise comparisons, p Value <0.05 was considered statistically significant.

Results

Figure 2

Based on the mean diameters,calcium hydroxide had the least zone of inhibition and combination of calcium hydroxide and chitosan had the highest zone of inhibition. All the measurement for zone of inhibition was carried out by a single examiner.

Groups	Zone Of Inhibition (mm)
$Ca(OH)_2$	8
CHITOSAN	18
PROPOLIS	22
Ca(OH)2 + CHITOSAN	30

Discussion

To increase the efficiency of instrumentation, root canal irrigating solutions and intracanal medicaments are used to eliminate the bacteria from the root canals.[12] Antibiotics are used in dentistry both systemically and topically.During systemic administration of antibiotics, negligible concentrations reach the root canal, whereas during the local administration of antibiotics, greater concentrations can be used as intracanal medicaments, to decrease systemic consequences and complications. Because of the complexity of root canal infection, single irrigant or a medicament or an antibiotic could not result in effective sterilization of the root canal. Combination of irrigants or medicaments decreases the development of resistant bacterial strains and produces synergistic effect, whose antimicrobial action lasts longer and also sustains release of medicaments occurs.[13]

Chitosan is a natural polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine.Chitosan is used for preparation of nanoparticles of various applications because of its biodegradable and nontoxic properties. It is insoluble in acidic conditions and free amino groups on its polymeric chain protonates and contributes to its positive changes. Mechanism of action of chitosan is thought to be that cationically charged amino group may combine with anionic components such as N-acetyl muramic acid, sialic acid, and neuramic acid on the cell surface and suppresses growth of bacteria by impairing the exchanges with medium, chelating transition metal ions, and inhibiting enzymes.[8] Therefore, chitosan has been added to Ca(OH)2 in an attempt to test the potential additive or synergistic effect on the viability of *E. Faecalis*.

According to the results of the study, propolis has better antibacterial efficacy. Mechanisms of propolis activity against microorganisms had been explained in a number of ways. The antibacterial action can be attributed to its flavonoid contents like quercetin, galangin, pinocembrin, esters of caffeic acid, benzoic acid and cinnamic acid [19]. In addition the ultraviolet absorbing component of propolis has been shown to inhibit bacterial DNA dependant RNA polymerase [20]. It is reported that propolis prevents bacterial cell division and act on the microbial membrane or cell wall site causing functional and structural damages, similar to the action of some antibiotics [21,22].

In this study, calcium hydroxide showed minimal antimicrobial effect compared with other medicaments. For calcium hydroxide to act effectively as an intracanal dressing, it should ideally occupy all the pulp space and should have close contact with the microorganism. Perhaps such contact does not occur in the total root canal system, where microorganisms can be located inside the dentinal tubules. Moreover, the low solubility and diffusibility of $Ca(OH)_2$, as well as the dentine buffering ability may make it difficult to attain an increased pH capable of eliminating bacteria located within dentinal tubules or enclosed in anatomical variations [6].Evans et al., demonstrated that the proton pump activity of *E. faecalis* offers resistance to high pH of calcium hydroxide [16].

Further studies are required to use these medicament combinations with chitosan as a drug carrier in *in vivo* studies.Great care has to be taken when administering antibiotics locally and also patient may have sensitivity to chemicals or antibiotics. The depth of penetration of the medicament combinations into dentinal tubules, their duration of action,concentration of the medicaments, and volume of the medicament to be given are to be investigated and compared.However, preclinical and clinical trials are required for the evaluation of biocompatibility and safety to recommend these intracanal medicament combinations for clinical usage.

Conclusion

Within the limitations of this study, it can be concluded that the combination of calcium hydroxide and chitosan showed better antimicrobial properties against *E.faecalis* than other medicaments.The therapeutic efficacy of antibacterial nanoparticles necessitate optimization of their physical, chemical, and biological characteristics, keeping in mind the tissue-specific factors at the site of infection and the method to deliver the NPs effectively in the target tissue.Therefore NP-based treatment strategies have the potential to improve antibacterial efficacy in endodontics and this whole concept of NPs in health care and endodontics should be accepted with positive zeal and caution in future developments.

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