Dental Research Journal

Original Article

Evaluation of the antibacterial effect of calcium hydroxide in combination with three different vehicles: An *in vitro* study

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ABSTRACT

Introduction: Antimicrobial activity of interappointment intracanal medications is an important consideration in endodontics. Considering the fact that calcium hydroxide (CH) cannot sterilize the root canal system, completing its antimicrobial spectrum seems necessary. The aim of this study was to compare the antibacterial activity of CH combined with three different vehicles in root canal system.

Materials and Methods: In this *in vitro* experimental study, 61 freshly extracted human single rooted teeth were used. After chemo-mechanical preparation, the teeth were dressed with CH in combination with: G1: Distilled water (DW); G2: 5.25% sodium hypochlorite; G3: 0.2% chlorhexidine solution. All teeth were mounted in a 2-chamber apparatus. After sterilization, the coronal chamber was exposed to bacteria and the apical chamber was filled with broth for 90 days. Leakage was recorded when turbidity was observed in broth. Mean times of leakage and turbidity percentage were recorded for each group. Data were analyzed by One Way ANOVA test (α=0.005).

Results: The highest mean time of contamination was for chorhexidine/CH combination (M=66.76 days), and the lowest was for DW/CH combination (M=40.29 days). Statistically significant difference was observed between G3 and G1 (*P*=0.042), but the difference between G2 and G3 (*P*=0.76) or G1 and G2 (*P*=0.18) were not significant. 88.23% of the samples of G1, 70.58% of G2, and 64.70% of G3 were contaminated after 3 months.

Conclusion: As an intracanal medication, the chlorhexidine/CH combination had significantly more antibacterial activity than DW/CH combination.

Key Words: Antibacterial effect, calcium hydroxide, chlorhexidine, intracanal medication, sodium hypochlorite

Received: October 2011
Accepted: January 2012

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INTRODUCTION

Microorganisms and their byproducts are considered the major causes of pulpal and periapical pathosis. The aim of modern endodontic treatment is the total elimination of microbes in the root canal system.[1] Bacterial elimination from an infected root canal system is a complex procedure, which is carried out by means of chemomechanical instrumentation and intracanal dressing.[2]

Calcium hydroxide (CH) has been an established antimicrobial medicament in dentistry for over 40 years,[3] and researchers have reported that it may be the best interappointment medication available against residual microbial flora.[4] When dissolved in water, CH dissociates into hydroxyl and calcium ions. The presence of hydroxyl ions alkalinizes a mixture resulting in antimicrobial characteristics.[5] Its high PH (approximately 12-12.5) has a destructive effect on bacterial cell membranes and protein structures.[5] However, CH is not equally effective against all

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types of endodontic bacterial infections. Entercocci are isolated in one third of the patients in whom endodontic treatment has failed.[6] Since enterococci are not eliminated efficiently by CH intracanal medication, it has been suggested to mix CH powder with antimicrobial endodontic irrigants to obtain a wider antimicrobial spectrum with a long lasting effect.[7] Different vehicles have been added to CH in an attempt to improve its antimicrobial activity, biocompatibility, speed of ionic dissociation, and diffusion. A variety of vehicles including aqueous, viscous or oily can be used for this purpose.[8] It has been shown that using viscous or oily vehicles may decrease the effectiveness of CH as root canal dressing.[5]

Sodium hypochlorite (NaOCl) and chlorhexidine digluconate (CHX) are antimicrobial agents frequently used in endodontics as irrigant as well as intracanal medicament.[9-11] Efficacy of CHX is because of interaction of the positive charge of its molecules with the negatively charged phosphate groups on microbial cell walls.[10] CHX has wide spectrum antimicrobial activity and prolonged action. Cervone *et al*.,[12] demonstrated that CHX has inhibitory effects on bacteria commonly found in endodontic infections. The association of CH and CHX has been used with encouraging result.[10,13,14] A number of studies using *in vitro* or *in vivo* models have stated that the antimicrobial efficacy of CHX/CH against E.faecalis is more than CH alone,[13-15] while others using different study designs have not found the same results.[16,17]

CH can also be prepared with NaOCl. In addition to its antibacterial properties, CH/NaOCl can effectively dissolute tissues and remnant debris in root canal system.[18] Moreover, the good compatibility of CH and NaOCl has been reported,[9,19] although the biologic consequences of endodontic application of this combination is still unknown.

CH combined with distilled water (DW) is currently the most commonly used mixture for intracanal medication. This is due to its properties such as fast ionic dissociation into alkaline ions, ease of use, cost effectiveness and also its ability to reduce osteoclastic activity and simulate repair.[8,20]

Estrella *et al*.,[21] stated that the type of vehicle has a direct relationship with the concentration and velocity of ionic liberation and therefore, with the antibacterial action of CH paste in a contaminated area. Despite numerous investigations, no vehicle has been proved to fulfill intracanal medicament expectations when mixed with CH, either biologically or clinically. The purpose of this study was to compare the antibacterial effectiveness of CH in combination with three different vehicles against E.faecalis in root canal system.

MATERIALS AND METHODS

In this *in vitro* experimental study, 61 intact caries free human single-rooted teeth with straight roots and mature apices were selected. The teeth were autoclaved and kept in 0.5% sodium hypochlorite (Merck, Germany) overnight. Crowns were removed using a bi-sided diamond disc to achieve an average of 13-15 mm root length. Then, patency of each canal was confirmed by inserting a size 10-kfile (Mani, Japan) through the apical foramen and the working length was established by subtracting 1 mm from this measurement. Root canals were rinsed with 5.25% sodium hypochlorite (Merck, Germany). Apical preparation was performed up to size 40-kfile (Mani, Japan) and completed by stepping back at 1 mm increments. Cervical and middle third preparation was performed using manual flaring up to size 80-Kfile (Mani, Japan).[22] The root canals were irrigated with 2 ml of 5.25% NaOCl between each instrument used.

After preparation, the root canals were irrigated with 5 ml 17% ethylene diaminetetraacetic acid (EDTA: Merck, Germany) and 5 ml of 5.25% NaOCl to remove the smear layer,[23] followed by a final flush of DW for total removal of NaOCl. The canals were dried with paper points before application of intracanal dressings.

Fifty-one instrumented teeth were randomly divided into 3 groups of 17 teeth each. Temporary dressings with 60 mg CH (Merck, Germany) mixed with 100 ml of three different vehicles were used, as follows:
G_1 = CH with DW,
G_2 = CH with 5.25% NaOCl,
G_3 = CH with 0.2% CHX (Behsa, Iran).

The intracanal dressings were placed with a size 35 Lentullo spiral (Danaher, USA). Temporary filling (Cavisol: Golchai, Iran) was placed at the orifices to avoid canal contamination. During all procedures throughout the experiment, the teeth were kept moist and in aseptic condition. Five teeth containing no intracanal medicament served as positive control (PC), and five teeth with one of the three pastes served as negative control (NC). Two coats of nail varnish were applied on the external surfaces of all teeth except for the 2 mm around the apical foramens

to prevent bacterial leakage through lateral canals or discontinuities in the cementum. All root surfaces of NC samples were covered with two coats of nail varnish.

The two-chamber apparatus used to evaluate leakage was prepared as previously described by Siqueira *et al*.[24] Each root sample was assembled in the testing apparatus and was sterilized overnight using ethylene oxide gas [Figure 1]. The whole apparatus was incubated at 37°C for 3 days to ensure sterilization. Then, the temporary filling was removed using a long shank sterile fissure bur (Tizkavan, Iran) with a sterile high-speed handpiece (MK-dent, Germany) under airflow hood. The upper chamber of each apparatus was filled with E.faecalis (ATCC29212) bacterial suspension and replenished every 3 days. Density of E.faecalis inoculum was adjusted to the turbidity of 0.5 McFarland standard (1.5×10^8 bacteria/ml).[25] The system was incubated at 37°C and checked daily for the appearance of turbidity in the lower chamber containing sterile Brain Heart Infusion broth (BHI: Oxoid LTD, UK) for the following 3 months. Leakage was reported when turbidity of BHI was observed. After observing broth turbidity, the growth of E.faecalis was confirmed by culturing the contaminated BHI in a blood agar broth and gram staining under light microscopy. Leakage data were analyzed with oneway ANOVA test to compare the mean day of leakage among all groups, and of each pair of groups. Significant level was set as 5%.

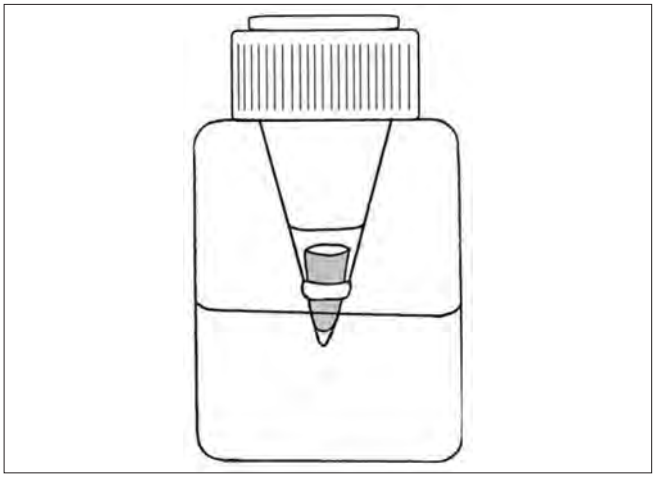

Figure 1: The dual-chamber leakage apparatus

RESULTS

No growth was observed when the sterilization of the whole apparatus was checked. All specimens of the PC showed broth turbidity within 1 day of incubation. The NC group showed no broth turbidity throughout the experiment. Leakage in experimental groups was first observed at the third day and displayed leakage within a range of 3 to 90 days. None of the groups were fully contaminated after 3 months of experiment. The number of leaking specimens per group and meantime of leakage in each group is presented in Table 1.

Paired comparisons showed significant difference among G_1 and G_3 (P=0.042), but no statistical difference was observed between G1 and G_2 (P=0.18) or G_2 and G_3 (P=0.76) groups. The turbidity percentage after 90 days for each group is presented in Table 1.

DISCUSSION

In addition to chemo-mechanical debridement, intracanal medications can be used to reduce the bacterial load and also to prevent coronal leakage during endodontic treatment.[1,3] These medications can act chemically by killing microorganisms that remain in root canal system or can act physically by preventing bacterial penetration. The present study was designed to compare the antibacterial effectiveness of CH in combination with three different vehicles in preventing bacterial leakage. The results of this study showed that CH/CHX and CH/NaOCl were more effective than CH/DW in preventing E.faecalis leakage. The difference between CH/CHX and CH/DW groups was significant. These findings indicate that the antibacterial potency of CH

Table 1: Pattern of bacterial leakage and turbidity percentage per group after 90 days of evaluation

Groups	Samples (*n*)	Leakage	No leakage	Turbidity percentage	Mean time of leakage
CH/DW	17	15	2	88.23	40.29
CH/NaOCl	17	12	5	70.58	59.29
CH/CHX	17	11	6	64.70	66.76
Positive control	5	5	0	100.00	-
Negative control	5	0	5	0.00	-

CH: Calcium hydroxide, DW: Distilled water, NaOCl: Sodium hypochlorite, CHX: Chlorhexidine

can be enhanced by preparing it with antibacterial irrigants such as CHX or NaOCl.

Leakage studies using bacterial cultures or human saliva have been used to test intracanal medications.[22,26,27] This method closely resembles clinical situation reproducing the relation between the tooth and the oral flora, and also allows sample evaluation at specific time periods. Bacterial leakage studies provide meaningful, precise and reproducible results.[28] However, contrary to clinical conditions, it is a static model; it needs extended periods of observation, and the amount of leakage cannot be correlated to the clinical outcome of the treatment.[27,29] Also, compared to bacterial suspensions, human saliva has proteins and enzymes which can interfere with the antimicrobial activity of intracanal dressings.[29]

Bacterial leakage studies have more biological and clinical relevance than dye leakage tests.[29] According to Siqueira *et al*.,[27] the dye leakage experimental model is subjective, less comparative, and with a low reproducibility. Dye leakage evaluations have several negative aspects: (i) dye penetration can be stopped by entrapped air; (ii) the size of the dye particles are less than that of bacteria; and, (iii) the dye can lose its color when it comes in contact with acidic solutions and some filling materials.[28]

Methods used in evaluating the antibacterial effect of different substances can produce conflicting results. Estrella *et al*.,[30] compared the antibacterial capacity of NaOCl and CHX using agar diffusion and direct exposure methods. The authors reported that NaOCl showed more antibacterial effect in direct exposure method and CHX showed more antibacterial effect in agar diffusion method. Agar diffusion method gives an inhibition zone around the discs containing the agent. In this method, the size of the microbial inhibition zone greatly depends on the solubility and infusibility of the test substance and therefore may not express its full potential. The direct exposure method, used in this study, is correlated to substance effectiveness in direct contact with microorganisms; it seems to be independent of other variables and appears to be a practical laboratory test. According to Estrella *et al*.,[31] this method would better simulate the real clinical conditions such as the presence of dentinal tubules, relationship between tooth and oral flora, and the relationship between the tooth and antibacterial agent.

The aim of combining CH with different vehicles is to enhance its antimicrobial effectiveness, particularly against resistant microorganisms such as E.faecalis found in failed root canal treatments. The antimicrobial activity of CH/CHX has been recognized by several studies[13,32,33] which support the findings of this research. However, Shafer *et al*.,[16] using a dilution method demonstrated that CH in association with DW had equal ability to prevent contamination of the system when compared to CH/CHX. This difference in results can be attributed to method of evaluation, experiment duration and the number of samples used.

The use of CHX is based on its ability to alter the osmotic equilibrium of bacterial cells. Haenni *et al*.,[34] reported that the antimicrobial activity of CHX is reduced when combined with CH, but CH did not lose its antimicrobial properties in such a mixture. Ercan *et al*.,[15] showed that 2% CHX gel was the most effective agent against E.faecalis inside dentinal tubules followed by a CH/2% CHX, and CHalone was totally ineffective even after 30 days. Nevertheless, CH has unique properties such as tissue dissolving capability, antimicrobial effect, maintenance in root canal for a long time and biocompatibility which cannot be ignored.[35] CH mixed with CHX can fulfill substantial antimicrobial requirements of an intracanal medicament such as the ability to eliminate E.faecalis, the most commonly isolated species from root canals of teeth with failed endodontic treatment.[36]

NaOCl is another irrigant with favorable antimicrobial activity. Valera *et al*.,[36] reported that 1% NaOCl was effective in reducing C.albicans and E.faecalis counts immediately after root canal preparation. However, the results of Verrisimo *et al*.,[37] study showed that NaOCl had the worst performance when used alone as intracanal medicament. The authors reported that this probably occurred because NaOCl becomes ineffective inside the canal within a short period of time and loses its antimicrobial properties. It has been found that NaOCl combined with CH shows equal antibacterial activity to CH/CHX.[9] This result was similar to the findings of the present study.

In disagreement with the present study, Zehnder *et al*.,[9] using direct exposure method on dentinal blocks, reported significant difference between CH/NaOCl and CH/DW. They indicated that dentin block disinfection was quicker and more thorough with CH/NaOCl than with CH/DW.

Unlike CHX, NaOCl can dissolve remnant debris in canal, a property which is desired from an intracanal medicament. However, NaOCl has limited capacity

to penetrate into dentinal tubules.[36] On the other hand, CHX has a property named substantivity which allows prevention of microbial colonization on dentine surface for some time beyond the actual period of time of medicament application.[38]

In conclusion, within the limitation of the present study, CH in combination with CHX showed significantly more antibacterial effect than its mixture with DW. The antibacterial activity of CH/NaOCl did not differ significantly from CH/CHX or CH/DW mixtures. Future research should focus on the biocompatibility of CH mixed with different vehicles. Also, further clinical studies can complete the findings of the present research.

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How to cite this article: Farhad AR, Barekatain B, Allameh M, Narimani T. Evaluation of the antibacterial effect of calcium hydroxide in combination with three different vehicles: An *in vitro* study. Dent Res J 2012;9:167-72.

Source of Support: This study was supported by a grant from the Vice Chancellor of Research of Isfahan University of Medical Sciences. This report is based on a thesis which was submitted to the School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran, in partial fulfillment of the requirements for doctoral degree in Dentistry (#389152). The study was approved by the Medical Ethics and Research Office at the Isfahan University of Medical Sciences and financially supported by this University., **Conflict of Interest:** None declared.