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RESEARCH: RESIDUAL ANTIBACTERIAL EFFECT OF CALCIUM HYDROXIDE COMBINED WITH CHLORHEXIDINE GEL AS AN INTRACANAL MEDICAMENT

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ABSTRACT

Introduction: Bacterial elimination from the root canal system is considered as an essential factor for a successful endodontic treatment. Aims: To investigate both in vitro and in vivo efficacy of calcium hydroxide paste (CH) combined with 2% chlorhexidine gel (CHX) at various time intervals on Enterococcus faecalis (E.f) when used as an intracanal medicament (ICM). Materials and Methods: For the in vitro study, 45 single-rooted teeth were used. After instrumentation, roots were infected with E.f and then divided into three groups (gps) (15 n/gp) according to the time interval for the medicament to be left inside the canal (1 week, 2 weeks and 3 weeks). ICM used was 2% CHX combined with CH (CH + CHX). At the end of each time interval, the antibacterial effect was evaluated using the agar diffusion test. For the in vivo study, 45 single-rooted teeth were selected. After sterilisation and access opening, the first pre-treatment sample was taken, then after instrumentation second post-instrumentation sample was obtained. Later, teeth were divided into 3 gps (15 n/ gp) (1 week, 2 weeks and 3 weeks) according to the time interval for the medicament to be left inside the canal. ICM used was 2% CH + CHX. After each period, the third post-medication sample was taken. ANOVA and Tukey post hoc tests were used for statistical analysis. Results: Both in vitro and in vivo studies have found that 2% of CH + CHX had a significant antibacterial effect for 1 week and 2 weeks. However, the antibacterial effect was significantly decreased after 3 weeks. Conclusions: CH + CHX can be used successfully for the reduction of E.f for 14 days when used as ICM. Keywords: Antibacterial, calcium hydroxide, chlorhexidine, Enterococcus faecalis, intracanal medicament.

Introduction One of the goals for the success of endodontic therapy is a complete bacterial elimination from the root canal to prevent reinfection since bacteria and their by-products are considered to be the primary cause of periapical lesions and failure of endodontic filling.[1] Various regimens have been used to decrease the numbers of root canal microbes including the use of instrumentation, irrigation and intracanal medications (ICM).[2] Biomechanical preparation of the root canals minimise endodontic infection but microbes can survive within the root canal system because of its anatomical complexity. Therefore, the use of biocompatible ICM having antimicrobial properties may reduce or eliminate bacteria in the root canal system and result in a significant increase in the success of endodontic treatment.[3] Calcium hydroxide (CH) is the most predominately used ICM in endodontics. The reason for its application is based on its mineralising properties and antimicrobial activity.[3,4] Iodoform has been incorporated in CH paste in an attempt to improve its antimicrobial properties.[5] Enterococcus faecalis (E.f) is the most common microorganism isolated from root treated teeth with the persistent periapical disease. This microorganism appears to be highly resistant to CH. So, there is a need for supplementary agents to effectively treat persistent periapical lesions.[6,7] Chlorhexidine (CHX) has an antibacterial effect against E.f, and the substantivity is the most effective feature of CHX, as it attaches to the hydroxyapatite of dentin, and this prolongs antibacterial efficacy.

Around 2% of CHX gel has been considered as the most effective ICM against E.f.[7,8] In some cases, during endodontic therapy CH had to remain in the root canal for a prolonged period. As it is ineffective against E.f, therefore, it must be combined with the antimicrobial agent that had prolonged antibacterial effect and effective against E.f to increase the success rate of endodontic treatment. Therefore, the purpose of the present study is to evaluate both in vitro and in vivo

residual antibacterial effect for a combination of CH and 2% CHX gel after different time intervals (1 week, 2 weeks and 3 weeks) when it is used as ICM. Materials and Methods In vitro study Specimens preparation About 45 fresh human teeth with uniradicular canal extracted for orthodontic reasons were used for this study. Teeth were stored in 0.1% thymol solution (BDH Chemical Ltd, England) at 4°C. The crowns were cut at the cementoenamel junction using carbide disc (KG Sorensen SP, Brazil) in a slow speed handpiece (W and H, Austria) with water cooling. Protaper Nickel Titanium Rotary system (Dentsply‑Maillefer, Switzerland) was used for instrumentation, using a rotary handpiece (Endo‑Mate DT, Japan). The rotation speed was adjusted at 250 rpm and torques 3 N/cm. Coronal 2/3 of the canal was enlarged using Protaper Shaping instruments S1 followed by S2. Preparation was finished using Protaper Finishing instruments F1, F2 and F3, respectively. Irrigation with distilled water was used during each file change. Apical 1/3 of the canal was cut in the same manner as described before. Root specimens with a length of 9 mm were prepared. In the apical and coronal sections of the specimens, a small cavity was prepared of about 1.5 mm in the depth and 2 mm in diameter. Apical cavities for all specimens were closed by acrylic resin (Newstetic.S.A, Colombia) to avoid bacterial leakage. After that, root canals irrigated with 5 mL of 5.25% sodium hypochlorite (NaOCl) (Chloraxid, medical company, Poland) and 1 mL of 17% EDTA (PD, Switzerland) which was left into the canal for 5 min to remove the smear layer. Finally, canals were given final irrigation with 5 mL of 5.25% NaOCl. Then, specimens were sterilised by autoclave (Hiayama MFG, CORP, Japan) at 121°C for 30 min. Each specimen was transported to brain heart infusion broth (BHI) (Oxiod LTD, Basingstoke, Hants/England), and incubated for 24 h at 37°C as a test for sterilisation. These specimens were then transferred to 3 mL sterile distilled water (SDW) in separate tubes for washing out BHI and to prevent contamination and dehydration and then incubated for 24 h at 37°C. After that, each root was removed from SDW and canals were dried using sterile paper points.

(Dentsply‑Maillefer, Switzerland) under aseptic conditions.[9,11] Contamination of the specimens The specimens stuck upright in sterile petri dishes using a quick setting epoxy resin (Eaglestar/ USA), were then infected with a standard volume of 10 µL (108 CFU) of E.f suspension (isolated from teeth with periapical lesions) and incubated at 37°C for 14 days. Every day fresh inoculum was added inside the canal to preserve the vitality of the bacteria. The specimens were removed from petri dishes and root canals were then irrigated with 5 mL of SDW, dried by sterile absorbent paper points.[9,11] Preparation of intracanal medicament CHX (chlorhexidine gluconate, Croatch Company) at 2% was used in this study, 2 g of orabase (NDA, Iraq) was added to 10 mL of the CHX to obtain a gel (pH 7.0). Orabase was checked in previous studies that had no antimicrobial effect.[8,9] CH paste (Metapex, Germany) was used in this study. CH paste was mixed with 2% CHX gel in a ratio of (3:1) (CH + CHX). Assessment of residual antibacterial efficacy All specimens were filled with CH + CHX paste by injecting it using a syringe and sealing the orifice with glass ionomer cement (Medicem, Germany). Then, the specimens were divided randomly into three groups of 15 samples according to the following period for each group as follows: 1‑week Group: ICM left inside the canal for 1 week. 2‑weeks Group: ICM left inside the canal for 2 weeks. 3‑weeks Group: ICM left inside the canal for 3 weeks. All specimens were stored in SDW and incubated at 37°C. After each time interval, the residual antibacterial effect was assessed using the agar diffusion test. An overnight mixed broth culture of E.f (108 CFU/mL) on brain heart infusion broth (BHI) was prepared. About 1 µL inoculum was inoculated into the E.f‑agar plate (Difco Laboratories Detroit Michigan, USA). For each agar plate, 4 F1 protaper files were placed on it, in which the first file was impregnated with fresh CH, the second file impregnated with fresh CH + CHX (positive control), the third file remained without coating (negative control), and the fourth file impregnated with the paste that was retrieved from the canal of each specimen. All plates were incubated into the incubator (Fisher Scientific, Russia) (37 ± 1°C) for 24 h, and then the inhibition zone was measured around each file at its largest diameter (mm) perpendicular to the file using digital vernia (Bosch, Germany).[12] In vivo study Patients' selection Patients included in this study were from those attending the Department of Conservative Dentistry, College of Dentistry, University of Mosul. Forty‑five patients with uniradicular teeth were included in this study. Their age ranged from 19–48 years and had no history of any systemic diseases. Teeth had an adequate coronal structure with necrotic or opened infected pulp in which pulp necrosis was diagnosed both radiographically and clinically. Patients were excluded from the study if they were on antibiotics therapy for 2 weeks before the treatment if the tooth was not suitable for rubber dam isolation, there was sinus opening, the initial culture showed no growth, if external and internal resorption was present, teeth with immature apex if it was impossible to reach the full length of the canal, and pregnant patients. Samples collection The tooth was isolated with a rubber dam (Digiflex, LTD Company, India). The tooth, its surroundings and the clamp (Ash, England) were disinfected with 70% ethanol (BDL limited pool, England). The carious lesion was removed, then the access cavity was prepared and the working length was detected using an apex locator (E‑PEX, Eighteenth, China). The pre‑treatment sample (S1) was obtained by inserting a sterile wet paper point into the canal and left for 1 min. Then the paper point was placed in the screw‑capped vial containing 5 mL BHI broth as transport media. Later, canal instrumentation was performed by Protaper Nickel Titanium Rotary system (in the same manner as described before) with irrigation

by normal saline (Sodium chloride 0.9%, Mosul, Iraq) to avoid any disinfecting action. Then, the post-instrumentation sample (S2) was collected in the same manner as for S1. This was followed by drying the canals with paper points and filling with CH + CHX paste by injecting it with a syringe and sealing the orifice with glass ionomer cement. Further, the canals were divided randomly into three groups with 15 samples for each group as follows

1 week Group: ICM left inside the canal for 1 week. 2-weeks Group: ICM left inside the canal for 2 weeks. 3-weeks Group: ICM left inside the canal for 3 weeks. At the end of each time interval, canals were passively re-instrumented with H-files (MANI-INC, Japan) and irrigated with SDW to remove ICM. Then, post-medication samples (S3) were obtained for each group from the canal as S1. Every vial containing the sample was transferred to the laboratory procedure within 1 h. Assessment of residual antibacterial efficacy Each vial of BHI broth containing the sample was shaken to disperse content evenly, 0.1 mL of it was taken by micropipette (Rainin, USA), cultured on Enterococcus agar and incubated into an incubator for 48 h at 37°C. Plates were tested and the number of colonies were counted. Statistical analysis The collected data were statistically analysed by SPSS version 19.0 for Windows software (IBM, USA) using one way ANOVA and post hoc Tukey tests. P value ≤0.05 was considered statistically significant. Results ANOVA test revealed a significant difference in the most tested group as present in Tables 1 and 2. In the in vitro study, from Table 1, Figures 1 and 2 results revealed that CH paste in negative control groups showed no inhibition zones in all tested groups. Results also found that the largest zones of inhibition were found around fresh CH + CHX which was significantly not different from CH + CHX retrieved from the canal after 1 week and 2 weeks, and significantly different from those retrieved after 3 weeks. However, the antibacterial effect of CH + CHX retrieved from the canal after 1 week and 2 weeks were nearly similar and significantly higher than CH + CHX retrieved from the canal after 3 weeks. In the in vivo study, from Table 2, Figures 3 and 4 results found that S1 and S2 for all groups showed no significant differences in the bacterial counts. The antibacterial effect of the 1-week group and 2-weeks groups were nearly similar and significantly higher than the 3-weeks group. Discussion Several reports revealed that E.f is a common isolate from infected root canals and persistent periapical lesions.[13] Therefore, they have been used in many studies for evaluating the antibacterial properties of materials because of their relative resistance.[7,9,11] CH containing materials have been predominantly used in endodontics for apexification, perforation repair, stimulate healing by hard tissue formation in root fracture, control external and internal root resorption, and has antibacterial action. CH is also the main component in root canal sealer and in several pastes that are used as intracanal dressings in case of periapical lesions.[4] Although CH is suitable as an ICM, it cannot be regarded as a universal ICM, because it is not effective against all microbes present in the root canal, especially E.f. However, the combination of antibacterial agents with CH should be avoided, particularly those that might be irritating to the periapical tissues.

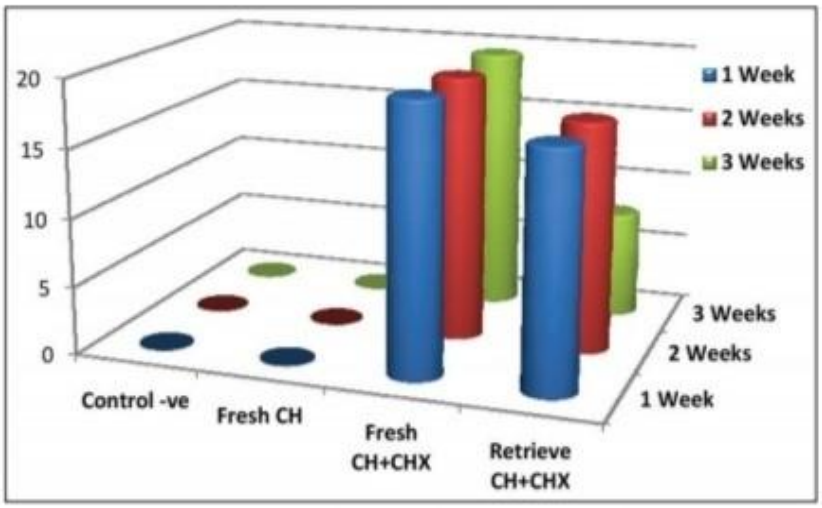

Figure 1: *In vitro* residual antibacterial effect of all tested groups

Table 1: *In vitro* antibacterial effect of the tested medicaments at different time intervals

Tested Medicaments and Groups		Inhibition Zone Mean (mm)±SD	F	*P**	Tukey
Fresh CH+CHX	1 week	19.6±1.07	0.081	0.922	C**
	2 weeks	19.4±1.17			C
	3 weeks	19.5±1.08			C
Retrieve CH+CHX	1 week	17.2±1.31	35.395	0.000	C
	2 weeks	16.9±1.28			C
	3 weeks	7.8±1.31			B
1-week Group	Control -ve	0±0	1737.415	0.000	A
	Fresh CH	0±0			A
	Fresh CH+CHX	19.6±1.07			C
	Retrieve CH+CHX	17.2±1.31			C
2-weeks Group	Control -ve	0±0	1612.216	0.000	A
	Fresh CH	0±0			A
	Fresh CH+CHX	19.4±1.17			C
	Retrieve CH+CHX	16.9±1.28			C
3-weeks Group	Control -ve	0±0	1403.069	0.000	A
	Fresh CH	0±0			A
	Fresh CH+CHX	19.5±1.08			C
	Retrieve CH+CHX	7.8±1.31			B

*$P \leq 0.05$ is statistically different. **The different letters vertically mean significant difference exists, similar letters vertically mean no significant difference exists. The comparison was done for each tested group and medicament alone. Number of samples for each group=15. ANOVA and Tukey post hoc tests were used for statistical analysis.

Table 2: *In vivo* antibacterial effect of the tested medicament at different time intervals

Samples and Groups		Bacterial counts Mean±SD	F	*P**	Tukey
Pre-treatment Sample (S1)	1 week	113.7±2.11	0.321	0.728	A**
	2 weeks	112.8±2.10			A
	3 weeks	114.5±2.06			A
Post-instrumentation Sample (S2)	1 week	105.8±1.70	0.012	0.988	A
	2 weeks	104.2±3.30			A
	3 weeks	102.1±2.55			A
Post-medication Sample (S3)	1 week	6.8±1.19	34.598	0.000	B
	2 weeks	11.6±2.13			B
	3 weeks	48.6±1.44			C
1-week Group	Pre-treatment Sample (S1)	113.7±2.11	796.256	0.000	A
	Post-instrumentation Sample (S2)	105.8±1.70			A
	Post-medication Sample (S3)	6.8±1.19			B
2-weeks Group	Pre-treatment Sample (S1)	112.8±2.10	577.230	0.000	A
	Post-instrumentation Sample (S2)	104.2±3.3			A
	Post-medication Sample (S3)	11.6±2.13			B
3-weeks Group	Pre-treatment Sample (S1)	114.5±2.06	620.533	0.000	A
	Post-instrumentation Sample (S2)	102.1±2.55			A
	Post-medication Sample (S3)	48.6±1.44			C

*$P \leq 0.05$ is statistically different. **The different letters vertically mean significant difference exists, similar letters vertically mean no significant difference exists. The comparison was done for each tested group and sample alone. Number of samples for each group=15. ANOVA and Tukey post hoc tests were used for statistical analysis.

Another medication, such as CHX gel which has a wide spectrum of antimicrobial effect (especially against E.f), biocompatible with periapical tissues, stays longer in contact with the microorganisms, diffuses through the dentin tubules and can be used in combination with CH to enhance its antimicrobial efficacy.[14-16] Therefore, the current study aimed to evaluate both in vitro and in vivo residual antibacterial effect for a combination of CH and 2% CHX gel after (1 week, 2 weeks and 3 weeks) when it is used as an ICM. In the present in vitro experiment, the agar diffusion test was used to assess the residual antibacterial effect. The sizes of inhibition zones depend on the diffusibility and toxicity of different components of the material into the agar. A fresh combination of CH + CHX was used as a positive control to compare it with the residual antibacteria effect of the material that is retrieved from the canals after 1 week, 2 weeks and 3 weeks. Besides, fresh CH without combination was used as a negative control to confirm that the CH did not affect E.f.

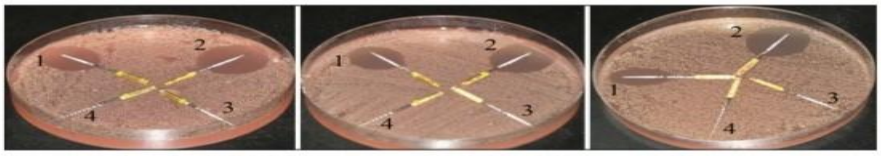

Figure 2: *In vitro* inhibition zones of all tested groups. A: 1-week group. B: 2-weeks group. C: 3-weeks group. 1: CH + CHX retrieve from the canal. 2: fresh CH + CHX. 3: fresh CH. 4: without any medicament

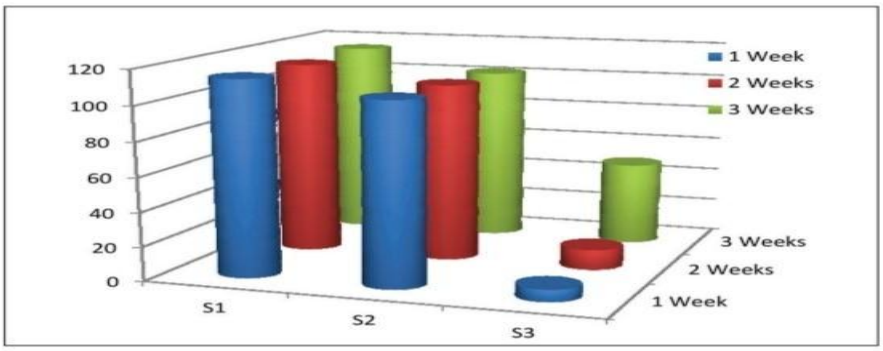

Figure 3: *In vivo* residual antibacterial effect of all tested groups

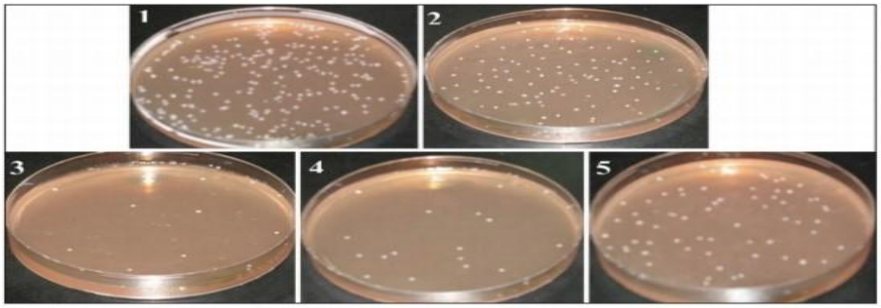

Figure 4: *In vivo* bacterial counts of all tested groups. 1: bacterial counts in pre-treatment sample. 2: bacterial counts in post-instrumentation sample. 3: bacterial counts after 1 week in post-medication sample. 4: bacterial counts after 2 weeks in post-medication sample. 5: bacterial counts after 3 weeks in post-medication sample

Therefore, the results detected that the largest zones of inhibition were found around fresh CH + CHX which was significantly not different from CH + CHX retrieved from the canals after 1 week and 2 weeks, and significantly different from those retrieved after 3 weeks. These results indicated that the addition of CHX to CH enhances the antibacterial effect for about 14 days because fresh CH showed no inhibition zone in all tested groups. Hence, this may be attributed to the higher CHX diffusibility in the agar media and potent antibacterial effect.[12,16] The present result is coinciding with several previous studies.[6,8,10,12,14,15] which reports that a combination of CH with CHX increases its antibacterial effect against E.f. However, it disagreed with Saatchi et al.[17] who showed that mixing of CH and CHX does not significantly increase the antimicrobial effect of CH against E.f., this may be related to the variation in the methodology, materials, time of the experiment and concentration of E.f. Besides, other studies comparing the efficacy of CH‑CHX combination to CHX alone against E.f., they were found that both were effective against tested microorganisms, and there is no significant difference between them.[18-20] Moreover, results found that there were no significant differences between materials retrieved from the canals after 1 week and 2 weeks, and the antibacterial effect of the material retrieved from the canal after 1 week and 2 weeks was significantly different from that retrieved after 3 weeks. This revealed that CH + CHX combination loses its antibacterial effect after 21 days, and maybe attributed to the fact that the component of every material used for a prolonged time may show a progressive reduction in the antibacterial activity. One should be related to that this material may be consumed in its biological and physicochemical properties, such as reaction with bacterial components which include protein and phospholipid.[12] After the assessment of the in vitro residual antibacterial effect for the CH + CHX in the current study, an in vivo study was performed to confirm its antibacterial effect. It was determined that S1 and S2 samples (bacterial counts before and after instrumentation) for all groups showed no significant differences, so this indicated standardisation for bacterial counts in all groups. Maximums reduction in the count of bacteria (S3) was observed in the 1‑week group and 2‑weeks group. The antibacterial effect of CH + CHX

related to the CHX as it has a wide antimicrobial spectrum effective against E.f destroys the bacteria by attaching to the bacterial cell wall. CHX acts by electrostatic interaction as it is positively charged and the bacterial wall is negatively charged, where interaction will occur and increase the cell wall coating permitting bacterial cytoplasm coagulation and lead to cell death. The prolonged antibacterial effect of CH + CHX may be due to the substantivity of CHX in which is adsorbed on the anionic substrate and then slowly released from these sites, thus resulting in long‑standing antibacterial effects.[6,19] Besides, the results of the current study revealed that the elimination of the E.f after 3 weeks is significantly decreased. This may be attributed to the buffering capacity of tissue fluid and dentine, reaction with enzymatic release from bacteria and dissolution of the material. Although it has been reported by the previous study that the addition properties (solubility, pH, flow and setting time) when used as an ICM.[7] In addition, Kontakiotis et al.[21] had been found that CH + 2% CHX can be used clinically without the disturbing sealing ability of root canal filling. Therefore, according to the findings of this study, we can conclude that CH + CHX can be used successfully for a reduction of E.f for 14 days, and when it is used for a period longer than this, it should be renewed since it loses its antibacterial effect. Conclusions Within the limitation of this study, the following conclusions can be drawn: 1. CH + CHX combination is effective against E.f both in vitro and in vivo. 2. CH + CHX has prolonged antibacterial effect for 14 days when utilised in the in vitro and in vivo studies. 3. CH + CHX loses the antibacterial effects after 21 days so it should be renewed when they are needed to be used for a period longer than 14 days.

Ethical Approval The work described in this study was approved by the Ethics Committee at the Department of Conservative Dentistry, College of Dentistry, University of Mosul, Mosul, Iraq. Also, informed consent was taken from each patient included in this study. Financial support and sponsorship Nil. Conflicts of interest There are no conflicts of interest.

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