

An in-Vitro Evaluation of the Antibacterial Activity of Experimental Chlorhexidine Gluconate Solution Using Agar Diffusion Test

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Key words

antibacterial, chx powder , agar diffusion test.

Abstract

The aim of this study was to investigate the antibacterial activity of three different concentrations of a newly produced CHX powder prepared locally in Mosul College of Dentistry in the Department of Dental Basic Sciences(approved by Nineveh Drug Industry (NDI,licence no.2256 in26/9/2002), and to compare it with the same concentrations of commercially available CHX solution against three bacterial isolates commonly isolated from infected root canals, using the agar diffusion test. A standard agar diffusion test was utilized to investigate the antibacterial activity of different concentrations of experimental chlorhexidine gluconate solution(NDI no.2256)and a commercially available chlorhexidine solution.The diameter of the microbial inhibition zones were measured in millimeter around each paper disk containing the experimental solutions. Data were analyzed using Duncan's Multiple Range Test ($\alpha = 0.05$). The results revealed that all tested microorganisms were affected by different concentrations of CHX powder(NDI no.2256) except for the lowest concentration (0.1%). Similar results were observed for CHX solution. The new prepared CHX powder(NDI n.2256)can be used a root canal irrigant.

Introduction

One of the goals of root canal treatment is to eliminate bacteria, bacterial products and debris from the root canal system ⁽¹⁾. Most bacteria found in the canal space may be removed by the mechanical action of endodontic instruments. However, in several situations, due to complex anatomy of the root canal system, organic residues and bacteria lodged deep inside the dentinal tubules can not be reached after careful mechanical instrumentation^(2,3). The use of irrigating

solutions is essential to ensure bacterial minimization^(4,5) and elimination of organic tissue remnants^(6, 7). Biomechanical cleaning and shaping of the root canal greatly reduces the number of bacteria. Nevertheless, because of the anatomical complexity of the root canal system, organic and inorganic residues and bacteria can not be completely removed and often persist ⁽⁸⁾. Various irrigants have been used during the canal preparation to minimize the residual debris, necrotic tissue, and bacteria, as well as to remove smear layer formed by the mechanical preparation of the dentin ⁽⁹⁻¹¹⁾. Chlorhexidine (CHX) is widely used in endodontic treatment as an irrigant. It has

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been emerged as a potential irrigant and inter -appointment medication to be used during the endodontic treatment of infected teeth. As an irrigant, CHX has shown antimicrobial effectiveness in several clinical and laboratory studies, although controversy exist ^(12,13). CHX solution lacks tissue-dissolving ability, less toxic to host tissues, and presents substantivity to dentin, which may results in residual antimicrobial effects for days to weeks ^(14,15). Chlorhexidine was developed in the late 1940s in the research laboratories of Imperial Chemical Industries Ltd. (Macclesfield, England). Initially, a sense of polybisguanides was synthesized to obtain anti-viral substances. However, they had little anti-viral efficacy and were put a side, only to be re-discovered some years later as antibacterial agents. Chlorhexidine was the most potent of the tested bisguanides ⁽¹⁶⁾. Chlorhexidine is a strong base and is most stable in the form of its salts. The original salts were chlorhexidine acetate and hydrochloride, both of which are relatively poorly soluble in water. Hence they have been replaced by chlorhexidine digluconate ⁽¹⁷⁾. Chlorhexidine is a potent antiseptic, which is widely used for chemical plaque control in the oral cavity. It is broad-spectrum antimicrobial agent that has been advocated as an effective medication in endodontic treatment ⁽¹⁸⁾. This solution, by attaching to bacterial cytoplasmic membrane, disrupts the osmotic balance, resulting in leakage of intercellular material. It also binds to hydroxyapatite and soft tissue, changing their electrical field to compete with bacterial binding ⁽¹⁹⁾. In addition, chlorhexidine gluconate presents a residual antibacterial effect on the infected canals ^(10,21). Chlorhexidine is highly effective against several gram-positive and gram-negative oral bacterial species as well as yeasts ⁽²²⁾. At high concentrations, CHX causes precipitation of intra-cellular constituents, particularly phosphate entities, such as adenosine triphosphate and nucleic acids. As a consequence, the cytoplasm becomes congealed, with resultant reduction in leakage, so that there is a biphasic effect on membrane permeability ⁽²³⁾. A new

locally prepared CHX powder has been recently produced in the Department of Dental Basic Sciences, College of Dentistry, Mosul University and approved by Nineveh Drug Industry(NDI),(license no.2265 in 26/9/2002), which justifies a study to compare it's antibacterial activity to that of a commonly used endodontic irrigant (CHX solution). The purpose of this study was to assess in vitro the antibacterial activity of this newly proposed CHX powder, and to compare the results with that of the most commercially available chlorhexidine glucuronate solution.

Materials and Methods

The in vitro antibacterial activity of the following materials against three reference isolates of bacteria was evaluated by the agar diffusion test :

Group I : A newly proposed CHX powder⁽²⁴⁾(NDI no.2256) in concentrations of (0.1%, 1% and 2%).

Group II : Chlorhexidine gluconate solution (4%) (Al-Rahma Pharmaceutical Co. Amman. Jordan) diluted to obtain concentrations of (0.1%, 1% and 2%). Normal saline (0.9 w/v sodium chloride) (Mosul I.V plant, Iraq) served as a negative control.

The following bacterial strains were used as indicator microorganisms in the study: Enterococcus faecalis, Staphylococcus aureus and Streptococcus mutants, all of which are commonly isolated from endodontically infected root canals ⁽¹⁰⁾. The same methodology proposed by Gomes et al.,⁽²⁵⁾ was used in this study. All microorganisms were previously subcultured in (appropriate culture) media and under gaseous conditions to confirm their growth. For the agar diffusion test, forty-two blood agar Petri plates were used for each strain of bacteria,i.e.(21) petri plate for Staphylococcus aureus and(21) petri plate for Streptococcus mutants for group I, and the same number was used for group II ,while for the Enterococcus Faecalis, forty-two (Enterococcus agar) Petri plates were used for group I and group II. The microorganisms were grown in 4 ml of

brain heart infusion broth (Oxoid LTD, Basingstoke, Hants, England) to enhance their growth at 37°C for 18 hours⁽²⁶⁾. To standardize the bacterial suspensions, the samples were diluted and counted to obtain a suspension of approximately 10⁹ (cfu/ml)⁽²⁷⁾. Then 7 replicates of blood agar plates (Oxoid LTD, Basingstoke, Hants, England, X 217) for each bacterial strain were inoculated with 0.1 ml of the adjusted bacterial suspension and spread on the surface of the agar using sterile swabs. Then (15 mm in diameter) sterile filter paper disks were dipped into each experimental solution and were applied over the surface of the agar after about 2 hours for pre-diffusion of the tested materials. Then the plates were incubated at 37°C for 48 hours⁽²⁶⁾. After the incubation period, the diameter of the bacterial growth inhibition zones around each paper disk was measured in millimeters. Data were analyzed using Duncan's Multiple Range Test ($\alpha = 0.05$).

Results

The results of the agar diffusion test are shown in Table (1) and figure (1). This table shows the diameters (mean and standard deviations SD) of the zones of bacterial growth inhibition (in mm) obtained for the tested materials. The values are expressed as means of 7 repetitions and SD. All tested microorganisms were significantly affected by the different concentrations of chlorhexidine gluconate solution. Similar results were observed for the experimental CHX solution (NDI no.2256), except for the lowest concentration (0.1%) which did not show any antibacterial effect. The largest growth inhibition zones were produced when the tested bacteria were in contact with 2% concentration for both tested solutions. Table (2) showed that there were no statistically significant difference ($P > 0.05$) between the growth, inhibition zones observed with the equal concentrations of CHX powder (NDI no.2256) and CHX solution (1%, 2%), though the solution form produced zones with diameters greater than the powder form. Also the diameter of the zones of

growth inhibition was greater for *Staphylococcus aureus* and *Streptococcus* mutants than for *Enterococcus faecalis* for both tested solutions.

Discussion

The present study investigated the antibacterial effect of three concentrations of experimental chlorhexidine powder (NDI no.2256) and compared the results with the same concentrations of chlorhexidine solution against *Enterococcus faecalis* which is the most commonly isolated strain of bacteria from root canals⁽¹⁾, and also *Streptococcus* mutants and *Staphylococcus aureus* which are also isolated from primary and secondary infected root canals and tooth caries⁽⁴⁾. Root canals of infected teeth have a complex flora consisting of cocci, rods, spirochetes, filaments and sometimes fungi. The common recovery of *Enterococcus faecalis* from the root canals of teeth in which previous treatment has failed is notable. *Enterococcus faecalis* have been shown to survive in root canals as a single organism without the support of other bacteria⁽²⁵⁾. This explains the resistance of this organism to low concentration of the experimental materials⁽²⁹⁾, i.e., 0.1% concentration of the powder form (NDI no.2256). Blood agar was used as the culture media, since this media is easily available and commonly used for the selected bacterial strains⁽³⁰⁾. All in vitro experimental methods have advantages and disadvantages. In the agar diffusion test, the size of the microbial inhibition zone depends upon the solubility and diffusibility of the test substance and may not express its full effective potential; therefore, the inhibition zones may be more related to material solubility and diffusibility in the medium. In addition to different other factors that may affect the reading of the results including: type of the medium, microorganisms, amount of inoculum and reading point of the zone of inhibition⁽²⁷⁾. The possible explanation of the difference in the antibacterial activity of the experimental materials is the selected isolates of microorganisms used,

and the method of measuring antibacterial activity^(31,32). In the present study, CHX powder (NDI no.2256) showed antibacterial activity against all tested microorganisms in 1% and 2% concentrations only. In 2% concentration: This material produced the largest mean zones of bacterial growth inhibition against *Streptococcus mutans* and *Staphylococcus aureus* (21 mm), followed by (18.5 mm) for *Enterococcus faecalis*. The mean zone, of bacterial growth inhibition produced by 1% of the solution form, were the following: 7.5 mm, 7.5 mm and 9 mm against *Enterococcus faecalis*, *Streptococcus mutans* and *Staphylococcus aureus* respectively. While the 2% concentration of the solution form produced a mean zones of bacterial growth inhibition and as follows : (22 mm, 27 mm and 26.5 mm) mean zones of growth inhibition against *Enterococcus faecalis*, *Streptococcus mutans* and *Staphylococcus aureus* respectively. There was no statistically significant difference ($P > 0.05$) between 2% concentration of both CHX forms, though the solution form produced zones with larger diameter than the powder form.

Also the difference was not significant ($P > 0.05$) between 1% concentrations of CHX forms, though the powder form produced zones with larger diameters than

the solution form. Although the difference was not significant, the results show that CHX in both powder and solution forms was less effective against *Enterococcus faecalis*. Emilson (1973), and Hennessy (1977) found that chlorhexidine is less effective on Gram-negative than on Gram-positive bacteria^(33,34). Many other ex-vivo studies used extracted human teeth mono infected with *Enterococcus faecalis*, a Gram-positive facultative species associated with failed root canal treatment and found the same results⁽³⁵⁾. The difference was significant between 0.1% and (1%, 2%) concentrations for both the solution the powder forms. 0.1% in the powder form was not effective against any of the test microorganism. This result suggests the use of CHX powder in a greater concentration. Aqueous solutions of 0.1 to 0.2% are recommended for chemical plaque control in the oral cavity⁽³⁶⁾, while 2% is the concentration of root canal irrigating solutions usually found in the endodontic literature⁽³⁷⁾. Based on the antibacterial activity of the tested solutions observed in the present study, it may be concluded that the experimental chlorhexidine gluconate powder (NDI no.2256) can be used as an alternative to chlorhexidine gluconate solution as a root canal irrigant.

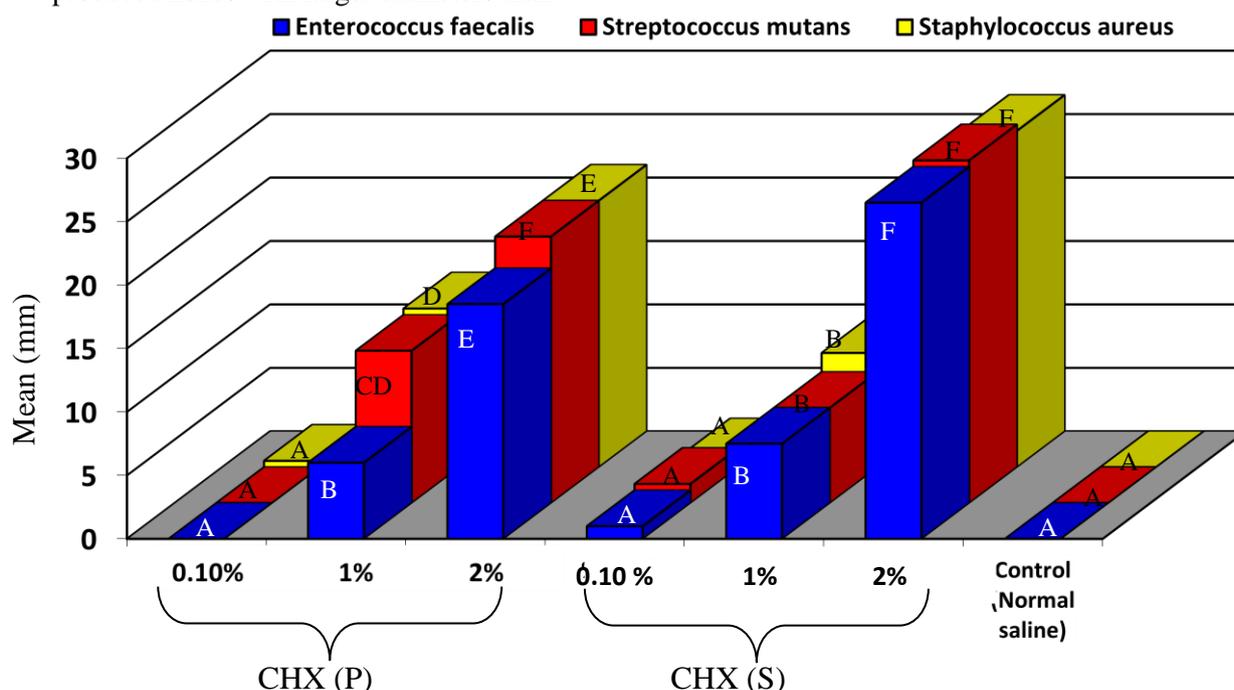


Fig.(1) :- Duncan's Multiple Range Test between diameters (in mm) of the zones of bacterial growth inhibition against each of the bacterial strains. (-- CHX (P): Chlorhexidine gluconate powder.-- CHX (S): Chlorhexidine gluconate solution. --Different letters horizontally or vertically mean significant difference at $p \leq 0.05$.)

Table (1):- Diameters (in mm) of the zones of bacterial growth inhibition against each of the bacterial strains.

Experimental materials	Enterococcus faecalis		Streptococcus mutans		Staphylococcus aureus	
	Mean	SD	Mean	SD	Mean	SD
*CHX (P)						
0.1%	0	0	0	0	0.5	0
1%	6	0.377	12	0.566	12.5	0.994
2%	18.5	0.556	21	0.449	21	0.645
**CHX (S)						
0.1%	1	0.243	1.5	0.267	1	0.377
1%	7.5	0.607	7.5	0.607	9	0.968
2%	26.5	0.393	27	0.748	26.5	0.393
Control						
Normal saline	0	0	0	0	0	0

* CHX (P): Chlorhexidine gluconate powder.

** CHX (S): Chlorhexidine gluconate solution.

The values are expressed as means of 7 repetitions and standard deviations (SD).

Table (2):- Duncan's Multiple Range Test between diameters (in mm) of the zones of bacterial growth inhibition against each of the bacterial strains.

Experimental materials	Enterococcus faecalis	Streptococcus mutans	Staphylococcus aureus
	Mean (Duncan's group)	Mean (Duncan's group)	Mean (Duncan's group)
*CHX (P)			
0.1%	0 (A)	0 (A)	0.5 (A)
1%	6 (B)	12 (CD)	12.5 (D)
2%	18.5 (E)	21 (F)	21 (E)
**CHX (S)			
0.1%	1 (A)	1.5 (A)	1 (A)
1%	7.5 (B)	7.5 (B)	9 (B)
2%	26.5 (F)	27 (F)	26.5 (F)
Control			
Normal saline	0 (A)	0 (A)	0 (A)

* CHX (P): Chlorhexidine gluconate powder.

** CHX (S): Chlorhexidine gluconate solution.

*** Different letters horizontally or vertically mean significant difference at $p \leq 0.05$.

References

- 1-Siqueira JF. Jr., Machado AG., Silveira RM., Lopes HP., Uzeda M. evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal in vitro. *Int.Endod.J.*,1997; 30:279-32.
- 2-Ferraz CC., Gomes BP., Zaia AA., Teixeira FB., Souza FJ., Filho. In vitro assessment of the antimicrobial action and the mechanical ability of chlorhexidine gel as an endodontic irrigant. *J. Endod.* 2001; 27(7): 452-5.
- 3-Thomas AM., Chandra S., Pandey RK. Elimination of infection in pulpectomized deciduous teeth short term study using iodoform paste. *J. Endod.* 1994; 20: 233-5.
- 4-Jeansonne MJ. White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. *J. Endod.* 1994; 20: 276-8.
- 5-Kuruvilla JR., Kamath MP. Antimicrobial activity of 2.5% sodium hypochlorite and 0.2% chlorhexidine gluconate separately and combined as endodontic irrigants. *J. Endod.* 1998; 24: 472-6.
- 6-Williams CECS., Reid JS. Sharkey SW., Saunders WP. In vitro measurement of apically extruded irrigant in primary molars. *Int. endod. J.*, 1995; 28: 221-8.
- 7-Kakehashi S., Stanley HR., Fitzgerald RT. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg. Oral Med. Oral Pathol.* 1999; 20: 340-9.
- 8-Peters OA. Current challenges and concepts in preparation of root canal system: a review. *J. Endod.* 2004; 30: 559-67.
- 9-Bystrom A., Sundqvist G. Bacteriologic evaluation of efficacy of mechanical root canal instrumentation in endodontic therapy. *Scand. J. Dent. Res.*, 1981; 89: 327-8.
- 10-Orstavik D., Haxpasalo M., Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules *Endod. Dent. Tramadol.* 1990; 6: 142-9.
- 11-Peters LB., Wesselink PR. Combination of bacterial species in endodontic infections. *Int. Endod. J.* 2002 ; 35: 698-702.
- 12-Ercan E., Ozekinci T., Atakul F., Gul K., Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: in vivo study. *J. Endod.* 2004; 30: 84-7.
- 13-Ruff ML. Mcclanahan SB. In vitro antifungal efficacy of four irrigants as a final rinse. *J. Endod.* 2006 ; 32: 331-3.
- 14-White RR., Hays GL., Janer LR., Residual antimicrobial activity after canal irrigation with chlorhexidine. *J. Endod.* 1997; 23: 229-31.
- 15-Rosenthal S., Spangberg L., Safavi K., Chlorhexidine substantivity in root canal dentin. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 2004; 98: 488-92.
- 16-Davies GE., Francis J., Martin AR., Rose FL., Swain G., Chlorophenyl diguanidohexane (Hibitane) ; laboratory investigations of new antibacterial agent of high potency. *Br. J. Pharmacol. Chemother.* 1954; 9: 192-6.
- 17-Foulkes DM., Some toxicological observations on chlorhexidine. *J. Periodontal Res. Suppl.* 1973; 12: 55-60.
- 18-Jeansonne MJ. White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. *J. Endod.* 1994; 20: 276-8.
- 19-Leonardo MR., Tanomara M. Filho, Silva LAB., Nelson P. Filho, Ito IY. In vivo antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution. *J. Endod.* 1999; 25: 167-71.
- 20-Oliveira DP. Barbizon JV., Trope M., Teixeira FB. In vitro antibacterial efficacy of endodontic irrigants against *Enterococcus faecalis* *Oral Surg. Oral Med., Oral Pathol. Oral Radiol. Endod.* 2007; 103(5) : 702-6.
- 21-Wang CS., Arnold RR., Trope M., Teixeira FB., Clinical efficiency of 2% chlorhexidine gel in reducing intracanal bacteria. *J. Endod.* 2007; 33(11): 128-9.
- 22-Vianna ME., Gomes BP., Berbe VB., Ferraz CC.,ZaiaAA.In vitro evaluation of chlorhexidine and sodium hypochlorite. *Oral Surg. Oral Med. Oral Pathol., Oral Radiol., Endod.* 2004; 97: 79-84.
- 23-McGurkin-Smith R., Trope M., Caplan D., Sigurdsson A., Reduction in intra canal bacteria using GT rotary instrumentation, 5.25% NaOCL, EDTA, and Ca(OH)₂. *J. Endod.* 2005; 31: 359-63.
- 24-Gaaffar A,Afflitto J,Nabi N:Chemical agents for the control of plaque and plaque microflora:An overview.*Eurp.J of oral sciences*,1997;105:2-5.
- 25-Gomes BPFA., Zaia AA., Teixeira FB., Souza-Filho FJ. In vitro assessment of the antimicrobial action and mechanical ability of chlorhexidine gel as endodontic irrigant. *J. Endod.* 2001 ; 27: 452-455.
- 26-Estrela C., Riberio RG., Estrela CR., Pecdra JD., Sousa-Neto MD., Antimicrobial effect of 2%

sodium hypochlorite and 2% chlorhexidine tested by different methods. *Braz. Dent. J.*, 2003 ; 14: 1-8.

27-Estrela C., Estrela CRA., Bammann LL., Two methods to evaluate the antimicrobial action of calcium hydroxide paste. *J. Endod.* 2007; 27: 5-7.

28-Sundquist G., Figdor D., Persson S., Sjogren U., Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg. Oral Med., Oral Pathol., Oral Radiol., Endod.* 1998; 85: 86-93.

29-Fabricius L., Dahlen G., Holm SE., Moller AJ., Influence of combinations of oral bacteria on periapical tissues of monkeys. *Scand. J. Dent., res.*, 1982; 90: 200-206.

30-Sipert CR., Hussne RP., Nishiyama CK., Torres SA., In vitro antimicrobial activity of fill canal, sealapex, mineral trioxide aggregate, Portland cement and endorez. *Int. Endod. J.*, 2005; 38: 539-543.

31-Estrela C., Riberio RG., Estcela CR., Pecdra JD., Sousa-Neto MD., Antimicrobial effect of 2% sodium hypochlorite and 2% chlorhexidine tested by different methods. *Braz. Dent. J.* 2003 ; 14: 1-8.

32-Michel AK., Nguyen TH., Chogle S., Antimicrobial activity of endodontic sealers on *Enterococcus faecalis*. *J. Endod.* 2003; 29: 257-258.

33-Hennessey TS., Some antibacterial properties of chlorhexidine. *J. Periodontal Res., Suppl.* 1977 ; 12: 61-7.

34-Emilson CE., Susceptibility of various microorganisms to chlorhexidine. *Scand. J. Dent. Res.*, 1973; 85: 255-65.

35-Siqueira JF., Ricas IN., Souto R., Colombo AP., *Actinomyces* species, *Streptococci* and *Enterococcus faecalis* in primary root canal infections. *J. Endod.* 2002; 28: 186-72.

36-Addy M., Moran JM. Clinical indications for the use of adjuncts to plaque control: chlorhexidine formulations. *Periodontal* 2000; 15: 52-4.

37-Zamany A., Safavi K., Spangberg LS. The effect of chlorhexidine as an endodontic disinfectant. *Oral Surg. Oral Med., Oral Pathol., Oral Radiol., Endod.*, 2003 ; 96 : 578-81