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ORIGINAL RESEARCH

An *in vitro* comparison of Antimicrobial Efficacy of Three Root Canal Irrigants—BioPure MTAD, 2% Chlorhexidine Gluconate and 5.25% Sodium Hypochlorite as a Final Rinse against *E. faecalis*

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ABSTRACT

Aim: This study was conducted to evaluate the antimicrobial activity of 5.25% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX) and BioPure MTAD when used as a final rinse against *Enterococcus faecalis.*

Materials and methods: Sixty single-rooted premolars were biomechanically prepared, inoculated with *E. faecalis* and divided into various groups. These were then irrigated with the test irrigants and tested microbiologically for growth of *E. faecalis* immediately after irrigation and after 48 hours.

Results: Statistical analysis showed that there was a significant difference between the antibacterial activities of BioPure MTAD, 2% CHX and 5.25% NaOCl at 5 minutes; however, the antibacterial activities of the three irrigants were comparable after 2 days of irrigation.

Conclusion: The present study concludes that BioPure MTAD is as effective against *E. faecalis* as 5.25% NaOCl and more effective than 2% CHX.

Clinical significance: *E. faecalis* is one of the most resistant intracanal species and a possible cause of root canal failure. Many authors have stressed the importance of using antimicrobial irrigants during chemomechanical preparation to ensure complete disinfection. Therefore, various irrigating solutions have been used during and immediately after root canal preparation to remove debris and necrotic pulp tissue and to eliminate microorganisms that cannot be reached by mechanical instrumentation.

Keywords: *Enterococcus faecalis,* MTAD, Sodium hypochlorite, Chlorhexidine gluconate, Antibacterial effect.

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INTRODUCTION

Microorganisms are the main cause of pulpal and periapical inflammation and the most common reason for root canal therapy. The primary aim of all endodontic procedures is to eliminate these infective microorganisms.[1] Enterococci are Gram-positive cocci that occur singly, in pairs or in short chains. They are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen. *Enterococcus faecalis* is associated with different forms of pulpal and periradicular diseases including primary and persistent endodontic infections.[2] According to Grossman, a thorough instrumentation and biomechanical preparation is the most important part of root canal therapy. The majority of bacteria found in the root canal microflora may be removed simply by the mechanical action of endodontic instruments.[3] However, due to the complexities of many root canals, even after mechanical procedures, organic residues and bacteria located deep in the dentinal tubules cannot be reached.[4] It has been shown that mechanical instrumentation without irrigation reduces but does not predictably eliminate bacteria in the canal.[8] These viable microorganisms remaining after root canal preparation contribute significantly to failure in endodontic therapy.[5] Many authors have stressed the importance of using antimicrobial irrigants during chemomechanical preparation to ensure complete disinfection. Therefore, various irrigating solutions have been used during and immediately after root canal preparation to remove debris and necrotic pulp tissue and to eliminate microorganisms that cannot be reached by mechanical instrumentation.[4]

Different antimicrobial agents have been introduced for disinfecting the root canal system; all these solutions have

certain disadvantages, such as limited antimicrobial activity, nonselectivity for host cells, inability to penetrate into dentinal tubules and a risk of allergy and toxicity in patients. Therefore, no ideal intracanal medication is available.

Various concentrations of sodium hypochlorite (NaOCl) have been used as root canal irrigants for many decades. The main advantages of NaOCl are its ability to dissolve necrotic tissues and its antibacterial properties against most microorganisms. Its main disadvantages are its unpleasant taste, high toxicity if extruded beyond the apex and its inability to remove the smear layer and kill all bacteria present in the root canal. Due to these limitations, a search for a better root canal irrigant continues. Chlorhexidine (CHX) gluconate, a cationic bisbiguanide, is a widely used mouthrinse in the prevention and treatment of periodontal disease and dental caries. It has been suggested as an irrigating solution in endodontics for its antibacterial effects and substantivity that results in a persistent antimicrobial effect for days to weeks preventing reinfection. BioPure MTAD–a mixture of doxycycline, citric acid and a detergent (Tween 80) has recently been introduced as a final irrigant for the disinfection of the root canal system. BioPure MTAD (MTAD) is less cytotoxic than 5.25% NaOCl. The results of some investigations have shown that MTAD can effectively remove the smear layer and abolish *E. faecalis*. The objective of this study was to evaluate the immediate and long-term antimicrobial activity of BioPure MTAD (a mixture of a tetracycline, acid and detergent) a relatively new root canal irrigant as a final rinse against *E. faecalis* and to compare it with 5.25% NaOCl and 2% CHX using a microbiological method.

MATERIALS AND METHODS

Sixty noncarious single rooted human premolar teeth with matured, closed apices, extracted for orthodontic treatment or periodontal disease were selected. The external root surfaces were debrided using a curette and all teeth were placed in 0.5% NaOCl for 24 hours for surface disinfection and stored in 0.9% sterile saline at room temperature until used. Teeth were subjected to standardized endodontic therapy. Conventional access cavities were prepared and biomechanical preparation was done using standardized technique to a master apical file size of #50. Sterile saline was used as irrigant during the procedure and the canals were finally flushed with 17% EDTA for 1 minute to remove smear layer followed by irrigation with saline. Subsequent to canal preparation, the apical foramina of all the teeth were sealed with cyanoacrylate adhesive to prevent leakage. The teeth were then mounted vertically in dental stone blocks to make both handling and identification easier.

The specimens were then autoclaved at 121°C and 15 lbs pressure for 20 minutes. Subsequent to sterilization, all the specimens were transported and manipulated under aseptic conditions using sterile instruments and equipment. Pure culture of *E. faecalis* (strain ATCC 29212) grown in brain heart infusion (BHI) broth for 24 hours at 37°C was used to contaminate the root canals. The beakers containing the sterile stone blocks in which the teeth were mounted were opened in a laminar airflow biosafety cabinet. An inoculum of 10 µl, of 24 hours pure culture suspension of *E. faecalis* was suspended into the prepared canals of all the teeth by using a sterile micropipette. The blocks were then placed inside sterile beakers and incubated at 37°C for 24 hours. The teeth were divided into the following groups according to the solutions used for irrigation:

Group A (n = 15) root canals were irrigated with 2 ml of 5.25% NaOCl for 5 minutes. Group B (n = 15) root canals were irrigated with 2 ml of 2% CHX gluconate for 5 minutes. Group C (n = 15) root canals were irrigated with 2 ml of MTAD for 5 minutes. Positive control (n = 10) root canals were irrigated with 2 ml of normal saline for 5 minutes and kept as positive control. Negative control (n = 5) root canals were not contaminated with *E. faecalis* suspension, i.e. they were sterile and were not exposed to any irrigating solutions (to confirm sterilization and the reliability of the procedures in the laboratory as to the absence of accidental microbial contamination). A volume of 2 ml of the test irrigant was used for each sample. Irrigants were allowed to remain in the canal for 5 minutes. Then a final irrigation was performed using 1 ml of sterile normal saline for each sample. Sterile paper points (ISO No. 45) were used to collect samples from the root canals—the points were inserted into the canal till working length, left for 10 seconds and transferred to tubes containing 5 ml of BHI broth. The tubes were vortexed for 30 seconds and incubated at 37°C for 2 days after which they were visually evaluated for occurrence of broth turbidity. Clear test tubes demonstrated complete sterilization and were designated as 'Cl' or 'Clear'. Blurred test tubes were considered as samples showing positive growth and labeled as 'T' (Turbid). Further samples were taken from turbid tubes using standard 0.001 Nichrome loops and subcultured on MacConkey agar plates and Pfizer enterococcus selective media and incubated at 37°C for 24 hours. These plates were evaluated to confirm the growth of *E. faecalis* and to rule out contamination. The plates on which growth occurred were labeled as 'PG' or 'positive growth' and those which did not show growth were labeled as 'NG' or 'negative growth'. Further tests—catalase test, growth in 6.5% NaCl, growth in broth at 9.5 pH, growth in bile-esculin agar and growth on potassium tellurite agar plates were carried out to confirm presence of *E. faecalis*.

The same irrigated specimens were incubated at 37°C for a further 48 hours and new samples using sterile paper points were collected at the end of 48 hours. This was done to test for and compare the long-term antibacterial effects of the irrigants. Paper points and irrigant controls were also incubated to rule out contamination.

RESULTS

Fifteen specimens were tested per irrigant. For the first set of samples (collected after 5 minutes of irrigation), bacterial growth was seen in one sample from the MTAD group (6.6%), 13 samples of the CHX group (86.6%) and three samples in the NaOCl group (20%). The second set of samples (collected after 48 hours) showed growth in one sample of the MTAD group (6.6%), six samples of the CHX group (40%) and one sample of the NaOCl group (6.6%) (Table 1). The negative controls showed no growth at any time during the study and the positive controls showed positive growth at all times. The results were statistically analyzed using Kruskal-Wallis test (Table 2). The p-values obtained show that there was a significant difference in the antimicrobial efficacy of the three irrigants, both at a 5-minute contact time and even after 2 days of irrigation. The pairwise comparisons between groups using the Mann-Whitney U-test (Tables 3 and 4) showed that both 5.25% NaOCl and MTAD have a comparable antimicrobial activity against *E. faecalis* after 5 minutes of irrigation. Also, both these irrigants have a significantly higher antimicrobial activity as compared to 2% CHX. However, there is no significant difference in the antimicrobial efficacy of the three irrigants after 2 days of irrigation. The Wilcoxon matched pairs test (Table 5) shows that there is no significant difference in the antimicrobial activities of 5.25 % NaOCl and MTAD at 5 minutes and 2 days of exposure time. However, the antimicrobial efficacy of 2% CHX is significantly greater at 2 days after exposure than at 5 minutes. Quantitative analysis was not performed since the colony counts were 10^5 CFU/mL or higher in all the cases.

Table 1: Mean of the three groups at 5 minutes and 2 days after irrigation

Groups	*5 minutes*			*2 days*		
	Mean	*Std. dev*	*Median*	*Mean*	*Std. dev*	*Median*
A (5.25% NaOCl)	0.20	0.41	0.00	0.07	0.26	0.00
B (2% CHX)	0.87	0.35	1.00	0.40	0.51	0.00
C (MTAD)	0.07	0.26	0.00	0.07	0.26	0.00

Table 2: Comparison of three groups at 5 minutes and 2 days after irrigation by Kruskal-Wallis test

	Groups	*Mean*	*Std. dev*	*Median*	*Sum of ranks*	*H-value*	*p-value*	*Significance*
5 minutes	A	0.20	0.41	0.00	285.00	22.9243	0.0000	
	B	0.87	0.35	1.00	510.00			S
	C	0.07	0.26	0.00	240.00			
2 days	A	0.07	0.26	0.00	307.50	7.43243	0.0243	S
	B	0.40	0.51	0.00	420.00			
	C	0.07	0.26	0.00	307.50			

Table 3: Pairwise comparison of the three groups at 5 minutes after irrigation by Mann-Whitney U-test

Groups	*Mean*	*Std. dev*	*Median*	*Sum of ranks*	*U-value*	*Z-value*	*p-value*
A	0.2000	0.4140	0.00	157.5000			
B	0.8667	0.3519	1.00	307.5000	37.5000	−3.1109	0.0019
A	0.2000	0.4140	0.00	247.5000			
C	0.0667	0.2582	0.00	217.5000	97.5000	−0.6222	0.5338
B	0.8667	0.3519	1.00	322.5000			
C	0.0667	0.2582	0.00	142.5000	22.5000	−3.7330	0.0002

Table 4: Pairwise comparison of the three groups at 2 days after irrigation by Mann-Whitney U-test

Groups	*Mean*	*Std. dev*	*Median*	*Sum of ranks*	*U-value*	*Z-value*	*p-value*
A	0.0667	0.2582	0.00	195.0000			
B	0.4000	0.5071	0.00	270.0000	75.0000	−1.5554	0.1199
A	0.0667	0.2582	0.00	232.5000			
C	0.0667	0.2582	0.00	232.5000	112.5000	0.0000	1.0000
B	0.4000	0.5071	0.00	270.0000			
C	0.0667	0.2582	0.00	195.0000	75.0000	−1.5554	0.1199

Table 5: Comparison of pre- and post-treatment scores in A, B and C groups by Wilcoxon matched pairs test

Groups	Time	Mean	Std. dev	Mean diff	SD diff	T-value	Z-value	p-value
A	5 minutes	0.2000	0.4140					
	2 days	0.0667	0.2582	0.1333	0.3519	0.0000	0.0000	1.0000
B	5 minutes	0.8667	0.3519					
	2 days	0.4000	0.5071	0.4667	0.5164	0.0000	2.3664	0.0180
C	5 minutes	0.0667	0.2582					
	2 days	0.0667	0.2582	0.0000	--	0.0000	0.0000	1.0000

DISCUSSION

It is well-established that pulpal and periapical diseases as well as failure of endodontic therapy are due to the presence of microbes in the root canal system. Eliminating microbes from the infected root canals and prevention of reinfection is one of the fundamental aims of endodontic therapy.[6] Of the *Enterococcus* species, *E. faecalis* is the most frequently isolated or detected species from oral infections, including marginal periodontitis, infected root canals and periradicular abscesses. *E. faecalis* is an unrelenting organism that, despite making up a small fraction of the flora in untreated canals, plays a major role in the etiology of persistent periradicular lesions after orthograde endodontic therapy. It has been hailed as the 'star survivor' and is commonly found in a lofty percentage of root canal failures. It thrives in treated root canals as a single organism or as a major portion of the flora. *E. faecalis* is a Gram-positive, catalase negative, fermentative, nonsporing facultative anaerobic, coccus.[7] Its cells are ovoid, ranging from 0.5 to 1 µm in diameter. These organisms occur singly, in pairs, or in short chains. Most strains are nonhemolytic and nonmotile. It has the ability to survive in various adverse circumstances. These comprise the capability to survive in hyperosmotic conditions, at temperatures ranging from 10°C to 60°C and at a pH of over 9.6. Growth on bile-esculin is a useful trait to identify Enterococci.[7] Studies have shown that these bacteria attain an increased resistance or phenotypic tolerance to numerous disinfectants or physical agents. Enterococci possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation and cause toxin-mediated damage. These factors include: (1) aggregation substance, (2) enterococcal surface proteins, such as esp, (3) gelatinase, (4) a cytolysin toxin, (5) extracellular superoxide production, (6) capsular polysaccharides and (7) antibiotic resistance determinant. They have shown resistance to calcium hydroxide and a number of intracanal medicaments.[8] Thus, an irrigant effective against *E. faecalis* is desirable. One of the most routinely used root canal irrigant today is NaOCl. It is a clear, straw-colored solution containing about 5% available chlorine. Beside their wide-spectrum, nonspecific killing efficacy on all microbes, hypochlorite preparations are sporicidal, virucidal and show a much higher tissue dissolving effect on necrotic than on vital tissues. Furthermore, NaOCl solutions are economical, easily obtainable, and have a good shelf life.[9] Irrigation of root canals with NaOCl solutions (in concentrations ranging from 1 to 5.25% is now a commonly established technique. Of all the presently used substances, NaOCl seems to be the most ideal, as it covers more of the requirements for an endodontic irrigant than any other known compound. However, the use of NaOCl has various inherent disadvantages, principally due to its toxicity, it injures all living tissues except keratinized epithelia. At very low concentrations, contact with vital tissues induces an inflammatory reaction. Accidental extrusion beyond the apex can cause excruciating pain, immediate swelling, profuse bleeding and also pharyngeal edema and esophageal burns when swallowed unintentionally. It may also damage the patient's clothing and eyes if splashed accidentally. NaOCl is extremely corrosive to metals; is strongly alkaline, hypertonic and has a foul odor and a very unpleasant taste.[5] The biocompatibility problems associated with the use of concentrated NaOCl have led to the use of substances with known antimicrobial properties and less toxicity, such as CHX or MTAD. CHX is probably the most widely used agent in antiseptic products; hand washing and oral products, and also as a disinfectant and preservative. It is an effective antiplaque agent and is regularly used in periodontal therapy and caries prevention.[9] CHX was developed in the late 1940s in Imperial Chemical Industries Ltd., Macclesfield, UK. It was Parsons and associates in 1980 who first studied the use of CHX as an effective endodontic irrigant.[10,11] It is a synthetic cationic bisguanide which is bacteriostatic at low concentration and bactericidal at high concentration. BioPure (Dentsply, Tulsa Dental, Tulsa, OK, USA), otherwise known as MTAD was introduced by Torabinejad and Johnson in 2003. This solution contains doxycycline hyclate (at a concentration of 3%), citric acid (concentration 4.25%) and a detergent, polysorbate 80 (concentration 0.5%, also known as Tween 80). Several studies have evaluated the effectiveness of MTAD for disinfection of root canals. Torabinejad et al have shown that MTAD is able to remove the smear layer and is effective against *E. faecalis*.[12] Doxycycline, has been used to remove the smear layer from instrumented root canal walls, for irrigation of retrograde cavities during

periapical surgical procedures and as intracanal medicament. Tetracyclines readily attach to dentine and are subsequently released without losing their antibacterial activity. This property creates a reservoir of active antibacterial agent, which is then released from the dentine surface in a slow and sustained manner. Thus, the presence of doxycycline may be accountable for the substantivity of MTAD. Doxycycline has also been shown to possess anticollagenase activity.[13] Citric acid in MTAD acts as a chelating agent and helps in removing the smear layer.[8] Polysorbate 80, the third component of MTAD is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid. The presence of a surfactant in the irrigating solution reduces its surface tension and increases wettability. This increases its protein solvent capability and enables better antimicrobial activity in uninstrumented areas of the root canal. Tween 80 facilitates a deeper penetration of the citric acid and doxycycline into the complex anatomic vagaries of the root canal space.[14,15]

Different approaches have been used to test the effectiveness of antimicrobial agents in the laboratory. These include: incubation of broth cultures of selected bacteria with the antimicrobial agent (O'Hara et al, 1993; D'Arcangelo et al, 1999), growth of selected bacteria as 'lawns' on agar surfaces and use of the disk diffusion method (Siqueira et al, 1998), the artificial infection of extracted teeth with selected bacteria and in-use irrigation with the test antimicrobial agents (Briseno et al, 1992; Sen et al, 1999). A combination of such approaches has sometimes been adopted but with discrepant findings (Shih et al, 1970; Foley et al, 1983).

In this study, prepared root canals were artificially infected with *E. faecalis* and a standardized irrigation protocol was followed. *E. faecalis* was selected because it is believed that it is an intracanal microorganism which is often associated with failed endodontic therapy and is one of the most resistant bacteria to elimination by disinfecting agents.

Apical enlargement during canal cleaning and shaping procedures has the potential to eliminate more bacteria from the root canal system. In the present study apical preparations were done up to 50 K-file in all the specimens. Flaring the canals with #50 K-file helped the irrigating solutions better penetrate into the canals so that lack of penetration of the solutions into the apical third of the canals would not be interpreted as lack of antimicrobial activity. The middle and coronal thirds were flared using Gates Glidden drills in a step back technique.

After bacterial inoculation, mechanical instrumentation of the canal was not performed, as the purpose of this study was solely to determine the antimicrobial effectiveness of the irrigants following a standardized irrigation protocol. A number of methods have been suggested for the collection of microbial samples from the irrigated specimens. These include the use of paper points collection of dentinal shavings from the internal root surface using files or external root surface using burs, pulverization of root tips in liquid nitrogen, etc. Various studies have reported the use of paper points for collecting microbiological samples from the root canals. Paper point cultures of the root canal detected bacteria more frequently than dentin filing cultures on files or reamers.[16] The root canals were irrigated with normal saline prior to obtaining samples to demonstrate the substantivity of the test irrigants and to prevent the carryover of the antimicrobial solutions. MacConkey agar and Pfizer Enterococcus selective media were used because they allow the growth of selective oral microflora, such as Enterococci including *E. faecalis*. Thus, the risk of false results due to the growth potential of bacterial contaminants, which might have occurred during handling, was reduced. Similarly, five specimens were used as negative controls and they were not contaminated with *E. faecalis* to ensure that sterility was maintained throughout the procedure and contamination did not occur at any stage during the experimental procedure. Normal physiological saline was used as a positive control irrigant as it was devoid of antibacterial action as compared to the other test solutions which had some amount of known antibacterial activity. Distilled water was not used as this would produce a hypotonic environment and lead to cell death due to endosmosis. A positive control was needed as only the antibacterial activity of the irrigant was under study and not the flushing action. All cultures obtained when normal saline was used as irrigant remained positive for *E. faecalis*.

Our study showed that there was a significant difference in the antimicrobial activity of the three root canal irrigants tested. At an exposure time of 5 minutes, both 5.25% NaOCl and BioPure MTAD had a comparable antimicrobial efficacy against *E. faecalis*, Furthermore, the antibacterial efficacy of both these irrigants was significantly greater than that of 2% CHX.

Even after 2 days of irrigation, the antibacterial activity of NaOCl and MTAD remained greater than CHX.

CONCLUSION

Within the limits of the present study, it can be concluded that the antimicrobial efficacy of BioPure MTAD is comparable to that of 5.25% NaOCl at both 5 minutes of contact time and 2 days after irrigation. The antimicrobial efficacy of BioPure MTAD is significantly greater than 2% CHX at 5 minutes of contact time. There is no significant difference in the antimicrobial efficacy of 5.25% NaOCl, 2% CHX and BioPure MTAD after 2 days of irrigation.

Antimicrobial activity is not the sole requirement of an endodontic irrigant. Root canal irrigants should also have

other characteristics, such as high detergent power, low surface tension, ease of handling and high proteolytic and tissue dissolving power. CHX does not possess pulp tissue dissolving ability[17] unlike NaOCl though there are reports of its tissue debriding capability. Until it is proven to be able to effectively dissolve pulp tissues, NaOCl must be still considered the irrigant of choice. CHX may still be used as an adjunct with NaOCl as an alternate endodontic irrigant due to its high bacterial adhesion reduction.

MTAD on the other hand has an antimicrobial activity comparable to NaOCl. It successfully removes the smear layer and has tissue dissolving property, but it is lower than that of NaOCl.[18] Thus, it may prove to be a successful replacement of NaOCl, however further *in vivo* studies are required before these laboratory results may be extrapolated to the clinical scenario.

Clinical Significance

E. faecalis is one of the most resistant intracanal species and a possible cause of root canal failure. Many authors have stressed the importance of using antimicrobial irrigants during chemomechanical preparation to ensure complete disinfection. Therefore, various irrigating solutions have been used during and immediately after root canal preparation to remove debris and necrotic pulp tissue and to eliminate microorganisms that cannot be reached by mechanical instrumentation.

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