ORIGINAL ARTICLE

Antimicrobial effectiveness of polyhexamethylene biguanide on *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Candida albicans*

Ivana Medvedec Mikić[1], Livia Cigić[2], Darko Kero[3], Danijela Kalibović Govorko[3], Goranka Prpić Mehičić[4], Arjana Tambić Andrašević[5], Paris Simeon[4]

[1]Department of Endodontics and Restorative Dental Medicine, [2]Department of Oral Medicine and Periodontology, Study Program of Dental Medicine, School of Medicine, University of Split, [3]Study Programme of Dental Medicine, School of Medicine, University of Split,Split,[4]Department of Endodontics and Restorative Stomatology, School of Dental Medicine, University of Zagreb, [5]Department of Clinical Microbiology, University Hospital for Infectious Diseases "Dr.Fran Mihaljevic"; Zagreb; Croatia

ABSTRACT

Aim To evaluate the antimicrobial activity of 0.2% polyhexamethilene biguanide (PHMB) in root canal models infected with *Enterococcus faecalis*, *Candida albicans* and *Staphylococcus epidermidis*. PHMB was compared in these tests with 2.5% NaOCl and 0.2% CHX.

Methods Prepared models of 50 human root canals (n=50) were immerged in mixed, four- weeks old culture that consisted of *E .faecalis, S. epidermidis* and *C. albicans*. Roots were randomly divided into three groups: one with 30 (n=30) and two with 10 (n=10) samples. Samples were treated with polyhexamethylene biguanide (PHMB) (0.2%), sodium hypochlorite (NaOCl) (2.5%) and chlorhexidine (CHX) (0.2%), respectively. Root dentin was sampled before and after the tretment with these solutions. Colony- forming units (CFU) were counted to asses the antimicrobial effects of three solutions on viability of selected microrganisms in specimens before and after the treatment.T-test was used for comparison of results between specimens before and after the treatment, while Newman-Keuls test was used for pairwise comparison at p=0.05**.**

Results The PHMB was significantly more efficient in reducing the number of all three tested microorganisms. NaOCl and CHX made only statistically significant ($p<0.05$) difference in case of *E. faecalis* and *S. epidermidis*. In the case of *C. albicans*, this difference was not statistically significant due to the small number of positive samples and high initial dispersion of results.

Conclusion Both solutions PHMB and NaOCl were successful in eliminating *E. faecalis* and *S. epidermidis* from the mature dentin biofilm, CHX was not successful enough.

Key words: antimicrobial agents, root canal irrigants, root canal preparation

Corresponding author:
Ivana Medvedec Mikić
Department of Endodontics and Restorative Dental Medicine,
School of Medicine, University of Split
Šoltanska 2, 21000 Split, Croatia
Phone: +385 21 557-846;
Fax: +385 21 557 800;
E-mail: ivana.medvedec@mefst.hr
ORCID ID:https://orcid.org/0000-0003-0202-3399

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INTRODUCTION

Bacteria (with their by-products) are predominant microorganisms found in infected root canals being organized in a microbial biofilm, which adheres to dentinal walls. Bacteria are also primary aetiological factors of pulp and periapical diseases. While some products of the bacterium may be directly linked to the damage of the periradicular tissues, a large part of the tissue damage is probably mediated by the host response to the bacterium and its products (1). Most of these microorganisms and their products are successfully eliminated during chemo-mechanical preparation of the root canal, which includes simultaneous use of various instrumentation techniques and antimicrobial irrigants (1). However, none of the root canal preparation protocols devised so far allows for complete root canal debridement (2).

Biofilm models have been studied in order to reproduce a polymicrobial infection *in vitro* (3). According to Thurlow et al.(4) a three-week old biofilm is considered to be a mature biofilm. *Enterococcus faecalis*, a Gram-positive anaerobic facultative coccus has been reported as one of the most frequently isolated microorganisms from the root canals at the time of retreatment (5). It is known to penetrate deeply into dentinal tubules (250- 400 μm) (6), binding to collagen and hydroxyapatite (7), which makes its complete elimination difficult. Because of these properties, *Enterococcus faecalis* has been adopted as a model test organism in many endodontic studies (8). *Candida albicans* yeast is larger than bacteria and can colonize dentinal tubules to depths approximately 150 μm (9) and has the ability to form biofilms even in nutrient-deprived conditions such as those in clean and filled root canals (10). It is the most frequently found fungal species in endodontically treated teeth with periradicular lesions (11). *Staphylococcus epidermidis*, a Gram-positive anaerobic facultative coccus, can form approximately 160 μm thick biofilm, which is resistant to antibiotics and antiseptics (12). Also an infection with *S. epidermidis* is followed with persistent symptoms in spite of using various intracanal antimicrobial agents and systemic antibiotic therapy (13).Until recently, it was thought to be a contamination from the skin, but Niazi et al. (14) showed presence of *S. epidermidis* in endodontic infections and connected it with failed endodontic treatments. Guimaraes et al.(15) in their research reported that *Streptococcus* spp. was the most isolated bacteria, followed by *Staphylococcus* spp.

Currently, sodium hypochlorite (NaOCl) is the most commonly used endodontic irrigant. Studies have demonstrated that 40- 60% of the root canals still have detectable levels of cultivable bacteria after chemo-mechanical procedures using NaOCl as the irrigant (16). Polyhexamethylene biguanide (PHMB) is a biocide of bisbiguanide family with a broad spectrum of use as disinfectant for surfaces, objects and instruments. It is also used in wound treatments (17), promoting wound healing (18), in mouthwash formulations (19) and in soft lenses care solutions (20). It is effective against Gram-positive (*S. epidermidis, S. aureus, E. faecalis*) and Gram-negative (*E. coli*) bacteria (21). On the other hand, chlorhexidine (CHX) has been well investigated as an endodontic irrigant for eradicating resistant bacterial strains (22), but it also showed good results as an antimicrobial agent against *Streptococcus* spp. (23) and has a good substantivity (24). However, CHX does have some adverse effects. It interacts with residual sodium hypochlorite in the root canal system resulting in a dark brown precipitate of parachloroaniline, which can change the tooth colour (25). Antimicrobial activity, ability to dissolve remnants of pulp tissue, lubrication during mechanical instrumentation, availability, and low cost are the fundamental requirements for root canal irrigants (26).

Since neither solution is ideal for root canal irrigation there is still need for the investigation of new irrigation solutions and techniques. Therefore, the aim of this study was to evaluate the antimicrobial activity of 0.2% PHMB in root canal models infected with *E. faecalis*, *C. albicans* and *S. epidermidis* in mixed, four- week old culture and compare it with antimicrobial activity of 2.5 % NaOCl and 0.2% CHX.

MATERIALS AND METHODS

Study design and materials

The study was performed during June and July 2015 at the Department of Clinical Microbiology, University Hospital for Infectious Diseases "Dr.Fran Mihaljevic" in Zagreb, Croatia. The aim of study was to evaluate the antimicrobial activity of 0.2% polyhexamethilene biguanide (PHMB) in root canal models infected with *Enterococcus faecalis*, *Candida albicans* and *Staphylococcus epidermidis*. The PHMB was compared in these tests with 2.5% sodium hypochlorite and 0.2% chlorhexidine. Specimens were extracted from human, single rooted teeth and they were divided in three groups according to irrigation solution used: 0.2% PHMB, 2.5% NaOCl, and 0.2% CHX.

The study was approved by the institutional Ethics Committee of the School of Dental Medicine, University of Zagreb.

Methods

Specimen preparation. In total, 50 human, single root, caries-free teeth without endodontic treatment, extracted for orthodontic or periodontal reasons were selected. Teeth were kept in 1% Chloramine (Pharmaceutical, Zagreb, Croatia) after the extraction. Curettages of root surfaces were performed and teeth were kept overnight in 0.5% NaOCl for disinfection. The crowns were cut off 1 mm from cement- enamel junction (CEJ) apically with 0.2 mm- thick diamond discs (Komet, Handelsagentur GmbH, Salzburg, Austria) and root shortened apically to standardize the root length to 10 mm. Each root canal was enlarged to size 40 with Hedström files (Dentsply Maillefer, Germany) while irrigating with 2.5% NaOCl. When the size 40 Hedström file fitted in the root canal (once the instrumentations of root canals were done), smear layer was removed using 17% EDTA (Ethylen Diamin Tetra-Acetat) for five minutes. The cement was also removed from the teeth and they were sterilized in autoclave for 20 min at 121°C. The outer root surface was coated with nail varnish and dried.

Microorganism inoculum preparation. The inoculum was prepared from *E. faecalis* strain (ATCC 29212), *S. epidermidis* (WHO 36) and *C. albicans* (ATCC 90028). Microorganisms were inoculated into tryptic soy broth (TSB) (Difco Labs, Detroit, MI, USA) and cultured under aerobic conditions at 37 ˚C for 24 h. The density was calibrated until turbidity reached 5-9x10^8 colony-forming units (CFU) per mL.

Specimen contamination and root dentine samples. Each specimen was immerged in tubes which contained 2 mL of broth and incubated in aerobic conditions at 37°C for 28 days. The culture medium was renewed every seven days according to the comparison results with control batch. In total 50 specimens were divided in three groups according to irrigation solution used: 0.2% PHMB, 2.5% NaOCl, and 0.2% CHX, one group with 30 specimens (n=30) and two groups with 10 specimens (n=10). After the incubation period, each specimen was irrigated with sterile saline (10 mL), dried with sterile paper point's size 35 (DiaDent, Burnaby, Canada), which were controlled for sterility. Using Hedström files size 45 by turning them 3 times clockwise fine dentin chips were collected and transferred into the microtubes with saline by placing Hedströmes into the microtubes. Aggregates were broken by ultrasound treatment. This represented specimens "A", or specimens before the treatment with antimicrobial solutions, which served as negative control. Subsequently, the first group (n=30) was treated with 10 mL of 0.2% PHMB (2 mL every two minutes) and then with 2 mL of neutralizer Tween 80 (Croda Health Care, Yorkshire, England, UK), dried with paper points and again using Hedstrom files size 45 in the same way as at specimens "A" fine dentin chips were collected and transferred in microtubes with saline. This represented specimens "B", specimens after the treatment with antimicrobial solutions. The second group (n=10) was treated with 10 mL of 2.5% NaOCl after which Tween 80 was used. The procedure was the same as for the first group of specimens. The third group (n=10) was treated with 10 mL of 0.2% CHX followed by irrigation with the same neutralizer and treated as described previously.

Microbiological analysis. 10-fold serial dilutions were performed from the microtubes with saline and dentin chips. To quantify the microbial growth, 0.1 mL aliquots were mounted on selective plates; Gelose D- Cocosel agar (bio-

Merieux, France), Mannitol Salt (MS) agar (Oxoid, UK) and Sabourand Chloramphenicol (SAB) agar (Bio- Rad, France) in triplicate. The plates were incubated at 37 ˚C for 48 h. CFU counts were then performed and log transforming of the CFU values was performed.

Statistical analysis

Data were statistically analysed using T-test for comparison between specimens "A" an "B" and Newman-Keuls test for pairwise comparisons. A $p \leq 0.05$ was considered as statistically significant.

RESULTS

In the post-treatment dentin chips samples there was evident decrease in the number of microorganisms (more than 50 times) for each of investigated microorganisms and solutions (Figure 1).

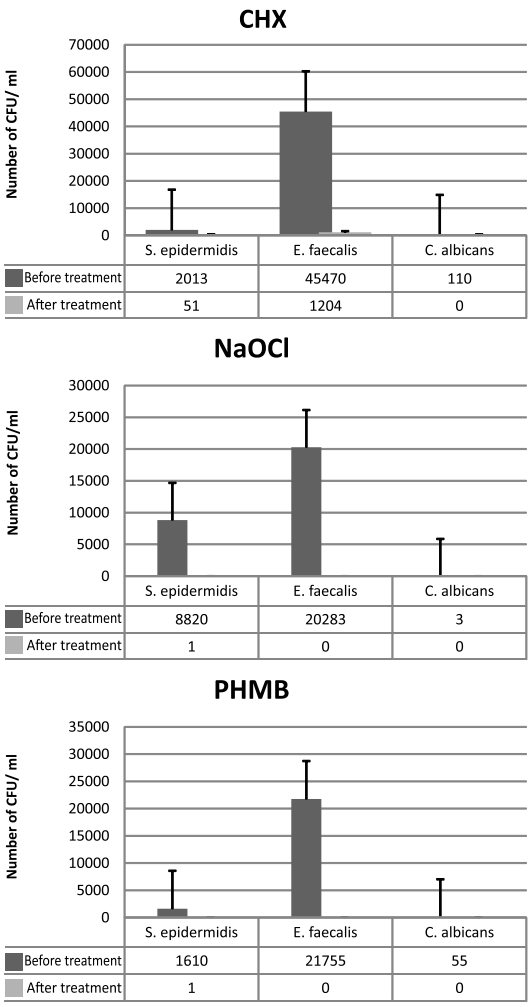

Figure 1. Mean value and standard deviation of the number of colony forming units per mililiter (CFU/mL) before and after the treatment with chlorhexidine (CHX), sodium hypochlorite (NaOCl) and polyhexamethylene biguanide (PHMB) antimicrobial solutions (statistically significant difference in the number of CFU/mL before and after the treatment in all three tested solutions)

The PHMB showed statistically significant ($p<0.05$) difference in the reduction of all investigated microorganisms (*C. albicans* p= 0.006, *S. epidermidis* p= 0.000 and *E. faecalis* p= 0.000). NaOCl and CHX also showed a reduction of the number of microorganisms between pre-treatment and post-treatment samples with *E. faecalis* (NaOCl p= 0.010, CHX p= 0.001) and *S. epidermidis* (NaOCl p= 0.010, CHX p= 0.001). In the case of *C. albicans*, the number of colonies before and after treatment with NaOCl and PHMB was not statistically significant due to a small number of positive samples and high initial dispersion of results (NaOCl p= 0.155, CHX p= 0.108) (Figure 1).

Since data on the number of colonies of microorganisms before the treatment was significantly different (Figure 1), the efficiency of microorganism removal was used to compare the antimicrobial activity of investigated antimicrobial solutions, rather than the number of colonies after the treatment. In the case of *S. epidermidis and E. faecalis,* PHMB and NaOCl showed almost the same efficiency, while CHX was less efficient, especially in the case of *E. faecalis*. Antimicrobial activity of all investigated antimicrobial solutions was the same for *C. albicans* (Figure 2).

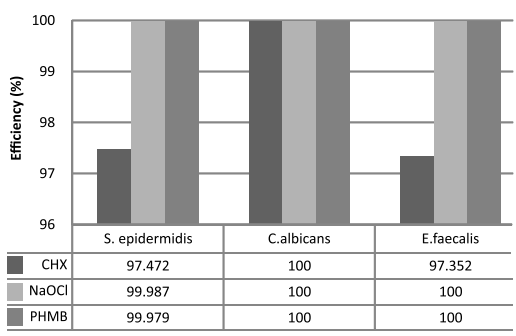

Figure 2. Efficiency (Mean value) of antimicrobial activity (%) for three different antimicrobial solutions and three microorganisms

A statistically significant difference ($p<0.05$) between investigated antimicrobial solutions for *Enterococcus faecalis* (p=0.001) but not for *S. epidermidis* (p=0.096) was found. For *C. albicans*it it was not possible to calculate the significance of

Table 1. The effectiveness of interaction between the three applied antimicrobial solutions (Newman-Keuls test)

	Staphylococcus epidermidis			*Enterococcus faecalis*			*Candida albicans*		
	CHX	**NaOCl**	**PHMB**	**CHX**	**NaOCl**	**PHMB**	**CHX**	**NaOCl**	**PHMB**
CHX		NS	NS		0.001	0.001		NS	NS
NaOCl	NS			0.001		NS	NS		NS
PHMB	NS	NS	NS	0.001	NS		NS	NS	

PHMB, polyhexamethylene biguanide; NaOCl, sodium hypochlorite; CHX, chlorhexidine; NS, non-significant

difference. Newman-Keulstest (Table 1) confirmed that there was significant difference between the antimicrobial effectiveness of CHX and NaOCl (p=0.001) and CHX and PHMB (p=0.001).

DISCUSSION

Data from this *ex vivo* study demonstrated the effectiveness of PHMB and NaOCl against *E. faecalis*, *S. epidermidis* and *C. albicans* in contrast to CHX.

There are numerous *ex vivo* studies on *E. faecalis* as a monospecies (5-8), but few of them are performed on mature, 4-week old biofilm consisting of bacteria *E. faecalis* and *S. epidermidis* and yeast *C. albicans*. The choice for microorganisms used in this study was based on microorganisms' level of virulence, resistance to antimicrobial agents and their prevalence in root canals after failed endodontic treatments. According to results obtained from de Lucenaet al. (27), 28 days is more than enough time for *E. faecalis* to penetrate 300 μm deep into the dentin. Also Haapasalo et al. (7) showed on in vitro model that after three weeks of incubation of *E. faecalis* a heavy infection was found 400 μm from the canal lumen, and the front of the infection reached 1000 μm in some blocks.

The culture method (CFU/mL) was chosen as a method for counting colonies after 4-week period of incubation and also the treatment with antimicrobial solutions. This method is sensitive enough to allow for exclusive detection of surviving microorganisms that are capable of dividing under favourable conditions (28). Furthermore, it was shown that this method and the quantitative real-time polymerase chain reaction (qPCR) can be reliably used for studies on antibacterial effectiveness (29).

Antimicrobial activity always presents a time dependent effect, especially in the case of NaOCl and CHX. Zandet al. (30) and Dumaniet al. (31) showed that even 1% NaOCl remains an effective agent for eliminating *E. faecalis* and *C. albicans* after one-minute treatment. Also, there are studies that support the notion that manual-dynamic irrigation is more effective than static irrigation and that the type of irrigation (dynamic/static) significantly influences the outcome of endodontic treatment (32,33).

Fine dentin chips were obtained from all specimens before (sample "A") and after (sample "B") the treatment so we could compare the effectiveness of three antimicrobial solutions.

That kind of collection samples (using Hedström files for collecting dentin chips) is limited to the main canal but according to Peters et al. (34) in layers closer to the pulp higher numbers of anaerobic bacteria and gram-positive rods were found, as well as a larger number of bacterial species.

The PHMB as a potent antimicrobial solution effective against Gram-positive and Gram-negative bacteria (35), yeasts (36) and viruses showed excellent results in eliminating *E.faecalis*. Although *E. faecalis* is well-known as a resistant endodontic bacterium with capability of surviving under harsh conditions (37-39), the number of its colonies was reduced after irrigation with 0.2% PHMB from 10^3 and 10^4 CFU/mL to 0 CFU/mL, respectively, which made the specimens sterile. Our study showed that 2.5% NaOCl showed similar results on *E. faecalis,* unlike the observations of Portenier et al. (40) and Liu et al. (28) showing that NaOCl was not efficient enough to eliminate *E. faecalis*. Kayaoglu et al. (41) and Chivatxaranukul et al.(42) suggested that *E. faecalis* resistance to NaOCl might be due to its binding affinity for collagen fibres and hydroxyapatite. 0.2% CHX was the least efficient antimicrobial solution in eliminating *E. faecalis*, which is in contrast with results of Oncag et al. (43) and Vianna et al. (44). In their studies, 0.2% CHX was able to eliminate *E. faecalis* within one minute of contact.

In case of *S. epidermidis*, both 0.2% PHMB and 2.5% NaOCl solutions were successful in eliminating this microorganism. Those results are in accordance with findings of Pappen et al. (45). These authors find that even 0.03% NaOCl was

successful enough in eliminating *S. epidermidis*. 0.2% CHX showed the same weak results as in the case of *E. faecalis*. Burgers et al. (46), Henderson et al. (47) and Brindle et al. (48) also demonstrated that CHX in various concentrations from 0.2% to 3.0% had no significant effects on *S. epidermidis*.

In the samples obtained after 4-week incubation in the same, mixed culture with *E. faecalis* and *S. epidermidis,* there was a very small number of colonies of *C. albicans.* Out of 50 specimens, 22 contained a very small number of colonies (10^1 CFU/mL), while in the rest of specimens there were no *C. albicans* colonies. According to Ning et al. (49) any *C. albicans* growing into dentinal tubules would not be easily removed for CFU counting. Some authors have suggested that yeast cells display better adherence than hyphae and the yeasts cells dispersed from biofilms also possess increased adherence (50), which might explain why it is difficult to remove *C. albicans* from the root canal. Therefore, data presented in this study do not allow us to make any solid conclusions on antimicrobial effectiveness of PHMB, NaOCl and CHX on *C. albicans* since the number of colonies of *C. albicans* in sample A was very low or zero.

The fact is that the faster growing species like *E. faecalis* could overgrew the other two species within 22 days of incubation but that is a real life situation.

In conclusion, PHMB and NaOCl substantially reduced the number of viable microbial cells (*E. faecalis* and *S. epidermidis*) in the tooth models with mixed, 4-week old biofilm, while CHX was not successful enough. The results present PHMB as a promising alternative for NaOCl.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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