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Research Article

ANTIBACTERIAL EFFICACY OF NISIN AS AN IRRIGANT AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM

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ABSTRACT

Objective: This study aimed to compare the antibacterial efficacy of 10% nisin, 2% chlorhexidine (ChX), and 2.5% sodium hypochlorite (NaOCl) against *Enterococcus faecalis* biofilm *in vitro*.

Methods: Petri dishes containing brain heart infusion agar were seeded with *E. faecalis* (ATCC 29212) and were incubated overnight at 37°C. The cellulose nitrate filter membrane was inoculated with *E. faecalis* for 72 h to grow a biofilm, and we performed the direct contact test between the test solutions and the biofilm for 10 min. The DNA was quantified using real-time polymerase chain reaction with propidium monoazide additive to count the living cells.

Results: The number of *E. faecalis* bacteria in the 2% ChX group was the lowest (8.36×10^3 CFU/mL) while the highest number of bacteria - among the antibacterial substances tested - in the nisin 10% group (5.55×10^6 CFU/mL).

Conclusion: The antibacterial effects against *E. faecalis* biofilm of 10% nisin were not comparable with those of 2% ChX and 2.5% NaOCl.

Keywords: Antibacterial efficacy, Biofilm, Chlorhexidine, *Enterococcus faecalis*, Nisin, Sodium hypochlorite.

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INTRODUCTION

Enterococcus faecialis is an organism often found in a failed endodontic treatment [1], at a level 9 times greater than that found in primary endodontic cases [2]. By bonding to the dentine and invaginating the root canal, *Enterococcus faecialis* is able to survive for a long time without any nutritive substrate; it has lysis enzyme, sitolisyn, agregatsubstant, feromon, and lipoteichoic uses the serum as a nutritive substrate, and competes with other cells, forming a biofilm [3-6].

The aim of endodontic retreatment is to eliminate bacterial infection and, particularly, the *E. faecalis* biofilm which is located in the root canal, to facilitate the healing process. Chemomechanical techniques in cleaning and shaping procedures mechanically prepare the root canal with instruments and support it chemically with an irrigating solution [7]. Therefore, an important aspect of cleaning the root canal is the irrigating solution in endodontic treatment [8]. Ideally, the irrigating solution should have an antibacterial effect without being toxic to the periapical tissue.

Some studies suggest that sodium hypochlorite (NaOCl) 2.5% and chlorhexidine (ChX) 2% have good efficacy against *E. faecalis*. However, Gomes-Filho *et al*. (2008) stated that NaOCl 2.5% and ChX 2% caused moderate inflammation to the periapical tissue, while NaOCl 5.25% was very toxic, causing severe inflammation to the periapical tissue [9,10].

Murray *et al*. (2007) identified the need for a non-synthetic irrigating solution which could clean and disinfect the root canal system yet has no toxic effect to the periapical tissue [9]. Hemadri *et al*. (2011) identified nisin as another irrigating solution with an antibacterial effect against *E. faecalis*. Nisin bacteria - produced by the *Lactococcus lactis* subspecies lactis - have been used as a preservative material for >50 years and are already claimed by the Food and Drug Administration as a generally recognized as safe substant [11].

A study conducted by Hemadri *et al*. (2011) showed that - when used as an intracanal medicament - nisin decreased *E. faecalis* by up to 49% while CaOH decreased *E. faecalis* up to 31% [11]. In our study, we aimed at analyzing the efficacy of nisin against *E. faecalis* compared to that of NaOCl and ChX, both of which have been widely used as irrigating solutions in endodontic treatment.

METHODS

This experimental laboratory study was conducted at the Laboratory of Microbiology and Biotechnology Center for Animal and Primate Studies, Bogor Agricultural University (IPB) from June to October 2014. As a research sample, this study used the bacterium *E. faecalis* (ATCC 29212), which we standardized with a 0.5 McFarland solution to obtain up to 108 CFU/mL bacteria. Then, the cellulose nitrate filter membranes (0.2 μm diameter size with 13 mm diameter, Whatman) were inoculated with 25 μL bacterial suspension, placed it on brain heart infusion agar (BHIA) (Neogen Corporation, Lansing, Michigan, USA) and incubated it at 370°C for 48 h in an aerobic state, to form biofilms.

The cellulose filter membrane was removed from the BHIA and added it to a 1 mL PBS solution to release the bacteria that were not firmly attached to the membrane surface. A cellulose filter membrane biofilm was inserted into tubes containing 1 mL PBS, 1 mL nisin 10% solution (N5764, Sigma-Aldrich, St. Louis, USA), 1 mL 2.5% of NaOCl, and 1 mL of CHx solution (Concepsis, Ultradent), and incubated them at 37°C for 10 min under aerobic conditions. Thereafter, the membrane was washed with 1 mL of PBS solution 3 times and centrifuged it for 2 min to release the bacteria attached to the membrane, to obtain a bacterial suspension. Then, the membrane was removed from the tube.

A solution of propidium monoazide (PMA) (PMATM dye 20 mM in water, Biotium) was added to each tube containing bacterial suspension, to a final concentration of 100 μM. Then, all the microcentrifuge tubes were incubated for 10 min at 40°C in a dark room. The microcentrifuge tube was placed horizontally on top of a storage box containing dry ice, and exposed it to 600 W halogen rays for 20 min, at a distance of 20 cm.

Table 1: Mean value of bacterial count (CFU/mL) after antibacterial agent exposure

Material test	Number of *E. faecalis*		95% CI	
	Mean (CFU/mL)±SD	Percentage	Lower limit	Upper limit
CHx 2%	8.36×10^{3}*±1.29×10^{4}	0.11*	-2.36×10^{4}	4.03×10^{4}
NaOCl 2.5%	$1.34\times10^{6}\pm1.09\times10^{6}$	0.99	-1.37×10^{6}	4.04×10^{6}
Nisin 10%	$5.55\times10^{6}\pm3.39\times10^{5}$	2.48	4.71×10^{6}	6.39×10^{6}
Control	1.66×10^{8}**±4.04×10^{7}	100**	6.57×10^{7}	2.66×10^{8}

The number of bacteria and the percentage of post-exposure with the highest test material (**) was the control and the lowest was CHX 2% (*). CHX: Chlorhexidine, SD: Standard deviation, CI: Confidence interval, NaOCl: Sodium hypochlorite, *E. faecalis: Enterococcus faecalis*

DNA extraction was performed by the thermal shock method. Our polymerase chain reaction (PCR) mix comprised Power SYBR®Green PCR Master Mix 10 µL, Primer EF Gro ES-F 2 µL, Primer EF Gro ES-R 2 µL, and nuclease-free water 2 µL. 16 Ml was added of this PCR mix to MicroAMP™ Fast Reaction Tubes (8 tubes/strip) and also added and homogenized 4 µL DNA samples with a micropipette. The MicroAmp™ Fast reaction tubes (8 tubes/strip) were covered with the MicroAmp™ Optical 8-Cap Strip asepsis. Then, the 48 PCR was inserted well plate into the Step-One Real-Time PCR System Applied Biosystem machine for DNA quantification.

The data obtained were statistically analyzed in the following way: To identify the antibacterial power of the test material on the growth of *E. faecalis* (CFU mL) bacteria, this study used the Shapiro–Wilk test to assess the data normality. The homogeneity of the data was analyzed based on the data after treatment using the non-parametric Kruskal–Wallis test, followed by the Mann–Whitney U-test significance test.

RESULTS

Table 1 presents the number of *E. faecalis* bacteria (CFU/mL) in the form of absolute quantification.

Table 1 shows that the number of *E. faecalis* bacteria in the 2% ChX group was the lowest (8.36×10^{3} CFU/mL) while the highest bacterial count was found in the control group (1.66×10^{8} CFU/mL). Compared with other antibacterial agents, we found the highest number of bacteria - among the antibacterial substances tested - in the nisin 10% group (5.55×10^{6} CFU/mL). Therefore, nisin had the lowest antibacterial effectiveness. However, when we compared nisin with the control group, we found the number of bacteria in the nisin group to be lower. Based on the percentages of live bacteria, we found that ChX had the lowest percentage at 0.11%, followed by NaOCl at 0.99% and nisin as 2.48%, against the control at 100%.

Table 2 presents the results of the Mann–Whitney U-test. The Kruskal–Wallis test delivered a p value of 0.016 ($p<0.05$). Therefore, we concluded that there were significant differences between the groups. From the results of the Mann–Whitney U-test, we concluded that the differences between the treatment groups were significant - that is, the nisin 10% and the NaOCl 2.5% group differed significantly ($p=0.05$), the nisin 10% group and the ChX 2% group differed significantly ($p=0.05$), the nisin 10% group and the control differed significantly ($p=0.05$), the NaOCl 2.5% group and the control differed significantly ($p=0.05$), the ChX 2% group and the control differed significantly ($p=0.05$), and the ChX 2% group and the NaOCl 2.5% group differed significantly ($p=0.05$).

DISCUSSION

This study used a pure strain of *E. faecalis* bacteria in this study (ATCC 29212TM). Since it has been used extensively as a representative control strain for clinical and laboratory research, *E. faecalis* (ATCC 29212) is commonly used in biofilm research [13].

In our study, we tested the antibacterial effectiveness of various agents on *E. faecalis* biofilm, because, often, the biofilm is found in the apical root canal system of the tooth with apical periodontitis and is an indication for root canal treatment. The biofilms that were used in this study were formed on cellulose nitrate filter membranes, which allow the growth of biofilms on standardized surfaces; this facilitated a more accurate assessment of the effectiveness of antibacterial agents [14].

Table 2: The significance value of the number of *E. faecalis* bacteria in the treatment group

Material test	Control	Nisin 10%	NaOCl 2.5%	CHx 2%
Control	-	0.05	0.05	0.05
Nisin 10%	0.05	-	0.05	0.05
NaOCl 2.5%	0.05	0.05	-	0.05
CHx 2%	0.05	0.05	0.05	-

Mann–Whitney U-test. *Significance test with $p\leq0.05$. CHX: Chlorhexidine, NaOCl: Sodium hypochlorite, *E. faecalis: Enterococcus faecalis*

When comparing the effectiveness of antibacterial agents against bacterial biofilms, it is important to standardize the age of the biofilms [15]. Santos *et al*. (2008) studied the morphology of *E. faecalis* biofilm on cellulose nitrate membranes for 24 h, 36 h, 72 h, 192 h, and 360 h. His results indicated that the 72-h biofilm had reached maturation. The biofilm showed the presence of EPS webs containing filaments and spaces formed from water-dried EPS in the bacterial cells, an indication of the early phases of growth and biofilm maturation [16]. Therefore, researchers used an *E. faecalis* biofilm matured for 72-h, which is a sufficient period of time to maintain an adequate biofilm density [14].

As test materials in this study, we used nisin 10%, NaOCl 2.5%, and ChX 2%. The choice of nisin 10% was informed by the study by Hemadri *et al*. (2011), which stated that the minimum bactericidal concentration of nisin was 10% [11]. According to Gomes *et al*. (2006), NaOCl 2.5% solution and ChX 2% could be successfully mixed with bacterial suspensions present in a single biofilm model formed on a cellulose membrane substrate [17].

NaOCl is the most commonly used irrigation material [18,19]. In an *in vitro* study conducted by Siqueira *et al*. (2000) to evaluate the reduction in post-instrumentation and irrigation bacteria with NaOCl 2.5% and 5.25% in a root canal inoculated with *E. faecalis* bacteria, they proved that both concentrations were effective in reducing the number of *E. faecalis* bacteria, but were not significantly different in their antibacterial effectiveness [20]. In this study, we used NaOCl 2.5% due to its good antibacterial power and lower toxicity compared to that of higher concentrations.

ChX, one of the antibacterial agents used in the field of endodontics, has a broad antibacterial spectrum for various organisms, such as *E. faecalis*. ChX in low concentrations is bacteriostatic (0.12%–0.2%), whereas at high concentrations it is bactericidal (≥2%) [20]. In this study, we used ChX at a 2% concentration, because this is a bacteriocidal dose and is commonly used as an irrigating solution for root canals.

In our study, we exposed bacteria to the test materials - NaOCl 2.5%, ChX 2%, and nisin 10% - for 10 min in an aerobic incubator at 37°C. Du *et al*. (2014) found that the effectiveness of antibacterial ingredients - namely NaOCl 2% and ChX 2% - against the bacterial biofilms in infected dentine was time-dependent. It takes at least 10 min to kill the bacterial biofilm in the infected dentine [21]. The residual viable bacteria on the disc were discharged using a centrifuge for 2 min, aiming to disrupt the biofilm structure and to obtain a bacterial suspension [22].

In this study, our molecular diagnostic method was PCR, which has advantages over other diagnostic methods. Some methods give an accurate estimation of the number and identification of bacterial species, with high sensitivity. However, this method is also capable of detecting live but non-cultivable bacteria - an important feature, because *E. faecalis* has this ability [23].

Reverse-transcriptase-PCR (RT-PCR) can detect all cells in an existing sample, including dead cells and other bacterial DNA in the environment, thereby over-estimating the result (with false positives). Therefore, in this study, we used PMA to detect living cells that had lost metabolic activity but still had intact cell membranes. This phenomenon also occurs in the viable but non-cultivable bacteria, which are detected by PMA as live bacteria but are unable to grow in agar culture [23].

Table 1 shows that nisin had 97.52% antibacterial effect while the control had no antibacterial effect. Based on the Mann–Whitney U-test we also proved that nisin differed significantly from the control, where the number of bacteria post-exposure to nisin decreased (5.55×106 CFU/mL) compared with the control (1.66×108 CFU/mL). This is in line with the study of Laukova *et al.* (2001), which stated that nisin had a bactericidal effect on broad-spectrum Gram-positive bacteria [24].

Based on our results, presented in Table 2, we found a statistically significant difference between antibacterial power among materials; the antibacterial efficacy of ChX 2% (99.89%) was significantly greater than that of NaOCl 2.5% (99.01%) and nisin 10% (97.52%), and the antibacterial effect of NaOCl 2.5% was greater than that of nisin 10%.

In accordance with a study of the bactericidal effects of NaOCl 2.5% and ChX 2% conducted by Vaziri *et al.* (2012), in which the amount of *E. faecalis* post-exposure to ChX was 8.2×109 CFU/mL, and that post-exposure to NaOCl was 8.6×109 CFU/mL, indicating that the bacterial content in ChX was lower than that in NaOCl; in addition, we found that ChX 2% showed better results than NaOCl 2.5% did [25].

In contrast to the results of this study, Spratt *et al.* (2001), in a study of the bactericidal effect of the exposure of *E. faecalis* biofilm grown on cellulose nitrate membranes to NaOCl 2.25% and ChX 2% for 15 min, found that NaOCl was more effective than ChX was [26]. This is at odds with the results of a study conducted by Mehrvarzfar *et al.* (2011), in which the antibacterial effect of NaOCl 2.5% and ChX 2% on root canals inoculated with *E. faecalis* differed significantly in the number of bacteria between NaOCl 2.5% and ChX 2% [27].

The methodology, bacterial characteristics of the biofilm, the time of exposure, and the concentration of the test material all influenced the differences in outcomes between these studies. Estrela *et al.* (2003) point out that differences in research methodology may influence the results of the study. His study compared the effectiveness of NaOCl 2% and ChX 2% against *E. faecalis* by direct contact and by the diffusion test method by calculating the inhibitory zone. NaOCl 2% showed better results than ChX 2% in the direct contact test did, whereas with the agar diffusion method, ChX 2% showed a larger inhibitory zone compared to that of NaOCl 2%. In the agar diffusion method, NaOCl 2% diffused into the solid agar medium. In the direct contact test, NaOCl 2% dissolved the organic material; therefore, it was in direct contact with the bacteria [28]. In addition, Nageshwar *et al.* (2004) asserted that ChX was likely to become inactive in the presence of organic material [20]. In this study, we used the methodology of direct contact between the *E. faecalis* bacterial biofilm and the test material, calculating the quantity of bacteria by quantitative calculation through RT-PCR; therefore, there was no reaction between the test material and the medium.

In this study, we found no test material - neither NaOCl 2.5% nor ChX 2% or nisin 10% - which could kill all bacteria completely. This is in contrast to previous research, which found that biofilms produced by *E. faecalis* inoculation in root canals for 24 h could be completely eliminated post-exposure to NaOCl 2.5% for 1 min, and ChX 2% for 30 min [28]. This difference can be attributed to the time factor of exposure, the method of creating the biofilm and the methodology. In our study, the RT-PCR method was used to detect live but non-viable, non-cultivable *E. faecalis* bacteria, which cannot grow in culture.

We found that the test materials had antibacterial power against *E. faecalis*. The antibacterial power of NaOCl is dependent on the concentration of hypochlorous acid. In solution, it may inhibit glucose oxidation, causing respiratory failure, and leading to cell death [19,29]. While ChX gluconate penetrates into the bacterial cell wall and causing the leakage of intracellular components. ChX, at high concentrations (2%), has a bactericidal effect due to precipitation or cytoplasmic coagulation caused by cross-linking protein [18]. Likewise, the bactericidal activity of nisin may cause bacterial lysis achieved by porous formation and inhibition of cell wall synthesis with a specific molecule, lipid II - a major component of the Gram-positive bacterial membrane of *E. faecalis*.

CONCLUSION

This study demonstrated the antibacterial power of nisin 10% against biofilm *E. faecalis*; however, its antibacterial action was less powerful than that of NaOCl 2.5% and ChX 2%.

CONFLICTS OF INTEREST

The author reports no conflicts of interest.

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