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Antimicrobial Activity of Different Irrigating Solutions against Selected Species Biofilms: An In-Vitro Study

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Abstract

Aim: The study was conducted to evaluate the antimicrobial efficacy of different endodontic irrigating solutions against selected species biofilms which are an integral part of endodontic microbial flora.

Materials and method: Single-species biofilms of Enterococcus faecalis, Staphylococcus aureus, Candida albicans and lactobacillus were generated on a cellulose nitrate membrane placed on agar medium. The biofilms were then immersed in the selected antimicrobial test agent for 30s and for 5, 10, 15, 30 and 60 mins, with and without mechanical agitation. Saline was used as control. After each time period, the membrane filters were then transferred to tubes containing 2 mL of fresh broth medium. The microorganisms were suspended using a vortex and the inoculum was serially diluted 10-fold. The plates were then incubated at 37 ºC under the appropriate gaseous conditions for 24 h (aerobes), 48 h (facultative anaerobes). The numbers of CFU per membrane were calculated. The tests were carried out in triplicate for each antimicrobial agent and microorganism and the survival curve was calculated. The samples were compared using the Friedman and Tukey test at a significance level of $P < 0.05$.

Results: The study showed that 2% CHX was the most effective antimicrobial agent followed by 3% NaOCl, NaOCl+ cetrimide and EDTA groups. The mechanical agitation improved the effectiveness of the antimicrobial agents, resulting in less time to eliminate the same micro-organisms. Saline did not inhibit the growth of any of the tested micro-organisms, with or without agitation, being statistically different ($P < 0.05$) from the rest of the tested irrigants.

Conclusion: Based on the results of the present study, it may be concluded that chlorhexidine solution has a great potential to be used as an intracanal auxillary chemical substance. Although sodium hypochlorite has been the most popular irrigating solution, other antimicrobial agents with less cytotoxicity should also be considered.

Keywords: Biofilm, Chlorhexidine, Sodium Hypochlorite, Surfactant.

Introduction

The primary aim of a root canal treatment consists of elimination of polymicrobial infections from the involved root canal system.1However root canal treatment may fail due to viable bacteria resisting treatment or microorganisms invading the canal via coronal leakage.[2] Also root canals with complex anatomy, limit the mechanical action of endodontic instruments and thus it is recommended to use the chemical solution with antimicrobial activity.3 The use of antimicrobial agents as adjuncts for irrigation and medication of root canals has shown to further reduce the bacterial counts.[4]

Infected root canals have a complex microbial flora that are along the root canal and may exists as loose collections in the moist canal lumen or as dense aggregates known as biofilm adhering to the dentinal wall.5A biofilm can be defined as communities' micro-organisms attached to a surface, embedded in an extra-cellular matrix of polysaccharides and it comprises of a protected mode of growth that allows survival in a hostile environment. Bacteria in such an environment constitute a highly resistant phenotypic state that is resistant to antimicrobial agents.

Sodium hypochlorite [NaOCl] is the most used endodontic irrigant that offers excellent antimicrobial activity against bacteria, fungi and biofilms6 and good tissue dissolving properties. The anti-bactericidal ability of sodium hypochlorite results from the formation of hypochlorous acid when in contact with organic debris. NaOCl exerts its effect by oxidation of sulfhydryl groups within bacterial enzyme system, thereby disrupting the metabolism of microorganisms.[7]

An alternative to NaOCl that has been proposed is chlorhexidine gluconate [CHX], primarily because of its substantivity.[4]CHX is a hydrophobic and lipophilic molecule that interacts with lipopolysaccharides and phospholipids on bacterial cell membrane, thus alters the osmotic equilibrium of bacterial cells.[8]

The sodium salts of EDTA are non-colloidal organic chelating agents, which can form non-ionic chelates with calcium. EDTA acts at neutral pH [between 7 and 8], thus believed to be more tissue friendly than other acid based demineralizing agents.[9]

One of the possible methods to improve the bactericidal efficacy of disinfecting solution is to incorporate different detergents as the surface-active agents that could help to reduce surface tension and increase wettability of solution. The reason for better killing of bacteria by the addition of detergents may be related to the weakening of cohesive forces in extracellular polymeric substances and bacterial membrane.[10]

Cetrimide is a cationic surfactant that has been often used in association with other irrigants during in root canal treatment.

Hence the purpose of this study was to evaluate the efficacy of irrigants mainly Sodium hypochlorite, Chlorhexidine, EDTA, Sodium hypochlorite+ Cetrimide on the selected species biofilms of Staphylococcus aureus, Candida albicans, Lactobacillus and Enterococcus faecalis.

Null hypothesis

HO (a): There is no significant difference between the efficacy of different antimicrobial solutions against selected species biofilms.

Alternate hypothesis

HO(b): There is significant difference between the efficacy of different antimicrobial solutions against selected species biofilms.

Materials and Method

In this study four root canal irrigating solutions namely Sodium hypochlorite, Chlorhexidine, EDTA, Sodium hypochlorite+ Cetrimide and four bacteria namely Staphylococcus aureus, Enterococococcus faecalis, Lactobacillus and Candida albicans were used.

Tubes containing 5 mL of BHI sterile suspension were inoculated individually with aerobe strains (C. albicans and S. aureus) and a facultative strain (E. Faecalis and lactobacillus). The suspension was then adjusted to match the turbidity of 1.5 × 108 CFU mL-1 (colony forming unit), which is equivalent to 0.5 McFarland standard.

Single-species biofilms of E. faecalis, S. aureus, C. albicans and lactobacillus were generated on a cellulose nitrate membrane (0.2-μm pore size and 47-mm diameter). The membranes were placed on the surface of 5% blood BHI agar plates (for aerobic and facultatively anaerobic micro-organisms) and further inoculated with 20 μL of each test micro-organism suspension. The plates, each containing four membrane filters, were incubated at 37 ºC again under the appropriate gaseous conditions: aerobes and facultative anaerobes in a CO2 incubator. The biofilm generation was observed visually and by SEM, where it was possible to verify the presence of biofilm after 10 days of incubation.

The membrane filters were aseptically removed from the agar plate and transferred carefully to tubes containing 5 mL of the selected antimicrobial test agent and saline for the control group, which were incubated for 30 sec, and also for 5, 10, 15, 30 and 60 mins with and without mechanical agitation. After each period of time, the membrane filters were then transferred to tubes containing 2 mL of freshly prepared broth medium. These were then vortexed for 30 s to re-suspend the micro-organisms. Tenfold serial dilutions were made up from the bacterial suspension and plated out on blood agar plate. The plates were then incubated at 37 ºC under the appropriate gaseous conditions for 24 h (aerobes), 48 h (facultative anaerobes). The number of Colony forming units [CFU] per membrane was calculated. The tests were carried out in triplicate for each antimicrobial agent and microorganism and the survival curve was calculated.

Statistical Analysis

Samples were statistically analyzed and CFU were calculated. Because of high SD of CFU means, samples were compared using the Friedman and Tukey test.

The p-value was compared with the level of significance. If $p<0.05$, then null hypothesis will be rejected and accept the alternate hypothesis. If $p>0.05$, the null hypothesis will be accepted. If there is a significant difference, multiple comparisons (post hoc test) will be carried out to find out among which group there exists a significant difference.

Results

Table 1 shows the contact time in seconds and the mean rank required for NaOCl, chlorhexidine, EDTA,

NaOCl+ Cetrimide and saline to produce negative culture against all microorganisms tested using mechanical agitation. Friedmann test showed that there was significant difference between the various antimicrobial solutions in reference to the contact time which depicted negative culture against selected species biofilms as mentioned in Table 1.

Table :1 Contact time in seconds and mean rank required for each irrigant to produce negative cultures against all tested microorganisms using the mechanical agitation

Bacterial Species	Contact time & Mean Rank					Mean Rank	P-Value
	NaOCl	CHX	NaOCl +Cet	EDTA	Saline		
E Faecalis	300(2)	30(1.5)	600(2.5)	3600(2.5)	3600(2.5)	2.9	0.004*
S Aureus	300(2)	30(1.5)	600(2.5)	3600(2.5)	3600(2.5)	2.9	
C Albicans	600(4)	300(3.5)	600(2.5)	3600(2.5)	3600(2.5)	3	
L Bacilus	300(2)	300(3.5)	600(2.5)	3600(2.5)	3600(2.5)	2.6	

Post hoc analysis revealed that the NaOCl and CHX was exhibiting a lesser contact time or improved antimicrobial activity individually in comparison to NaOCl+ Cetrimide, EDTA and saline group, which was statistically significant at p value< 0.046.

Table 2 shows the contact time in second and the mean rank required for NaOCl, chlorhexidine, EDTA, NaOCl +Cetrimide and saline to produce negative culture against all microorganisms without mechanical agitation. All microorganisms were killed within 300sec (5 minutes) by CHX solution. Candida albicans took 600 sec to be killed by NaOCl which was the most resistant microorganism to 3% NaOCl. No statistically significant difference was observed between NaOCl and CHX group. All microorganisms tested survived upto 600 sec (10 minutes) by NaOCl + Cetrimide group, whereas EDTA alone took almost 3600sec (60 mints) to kill all the microorganisms. However, all of them were statistically different ($p<0.005$) from the control group.

Comparing the values from table 1(treatment with mechanical agitation) with the values from table 2 (without mechanical agitation), it can be observed that mechanical agitation promoted the effectiveness of the antimicrobial agents resulting in less time to eliminate the same microorganisms.

Table :2 Contact time in seconds and mean rank required for each irrigant to produce negative cultures against all tested microorganisms without mechanical agitation

Bacterial Species	Contact time & Mean Rank					Mean Rank	P-Value
	NaOCl	CHX	NaOCl +Cet	EDTA	Saline		
E Faecalis	30 (1.5)	30 (2)	300 (1.5)	3600 (2.5)	3600 (2.5)	2	0.003*
S Aureus	30 (1.5)	30 (2)	300 (1.5)	3600 (2.5)	3600 (2.5)	2	
C Albicans	300 (3.5)	30 (2)	600 (3.5)	3600 (2.5)	3600 (2.5)	2.8	
L Bacilus	300 (3.5)	300 (4)	600 (3.5)	3600 (2.5)	3600 (2.5)	3.2	

However, saline did not inhibit the growth of any of the tested microorganisms with or without agitation being statistically different ($p<0.05$) from other antimicrobial solutions.

Discussion

The goal of cleaning and shaping regimen in endodontic therapy is to maximally reduce microbial load and necrotic tissue remnants in the root canal system. Mechanical instrumentation leaves around 40-50% of the root canal walls untouched. Irrigating solutions are therefore required in conjunction with mechanical preparation for optimal results.[9]

Bacteria growing in the biofilm may survive starvation periods and recover rapidly and may exhibit new and more virulent types. Furthermore, bacteria within biofilms have inherently increased resistance to antimicrobial agents compared with the same bacteria grown under planktonic condition. The antimicrobial efficacy of endodontic irrigants is tested in vitro with the use of planktonic cultures. Biofilms contain organization of bacteria that confer to a range of phenotypic properties that are not evident in their planktonic counterparts and confer a reduced susceptibility to antimicrobial agents. Therefore, the use of a biofilm

model could reproduce more precisely the in vivo conditions.[11]

The biofilm model in this study was used to evaluate the antimicrobial efficacy of substances used during the chemo-mechanical preparation against selected microorganisms commonly found in root canals. The methodology performed was based on the one used by Spratt et al. (2001), which allows the biofilm to grow on cellulose nitrate membranes. The antimicrobial agent was tested in direct contact with a single biofilm.[11]

The strains used in this study were E. Faecalis, Lactobacillus, Candida albicans and Staphylococcus aureus as they are an important part of endodontic microbial flora.

Enterococcus faecalis, a facultative anaerobic Gram-positive coccus has been implicated in persistent root canal infections and has been used in several previous studies on the efficacy of endodontic irrigants especially due to its high level of resistance to antimicrobial agents. In the present study we used ATCC strain because it was also utilized in previous in vitro studies investigating the antibacterialeffects of endodontic irrigants (Siqueira et al. 1997, 1998a, Gomes et al. 1999, Ferrazet al.). Moreover, it has been stated that E faecalis is one of the most common microorganisms found in the endodontic failure cases.[12]

Biofilms on both inanimate and biological surfaces are readily formed by Candida Albicans. Moreover, it is the fungal species most isolated from infected root canal[11]. Sen et al have been demonstrated that C albicans can grow on the root canal walls in different forms in a nutritionally stressed medium and can penetrate the dentinal tubules. According to Nair et al candida has been implicated in endodontic flare ups and has been studied extensively with respect to the efficacy of different endodontic irrigants.[13]This was one of the reasons to select Candida as a strain for this study along with E faecalis. Likewise, staphylococcus and lactobacillus also induce acute dental infection and are an integral part of endodontic microbial flora.

NaOCl has a wide variety of applications as a root canal irrigant at different concentrations. Laboratorial and clinical investigations have shown that NaOCl produces an effective chemo mechanical debridement of the root canal system, due to its properties, such as lubricating action for instrumentation, antimicrobial activity and dissolution of pulp tissue. However, the usage of NaOCl in high concentrations is unacceptable because of its irritating effect on the periapical tissues. Therefore, in this study 3% NaOCl has been used to investigate its antibacterial efficacy against selected species microorganisms.[3]

Chlorhexidine gluconate is a cationic bisguanide that acts on the cell wall of the microorganism causing leakage of intracellular components. At low concentrations, chlorhexidine has a bacteriostatic effect, causing the leaching of small molecular weight substances from microorganisms. At higher concentrations, chlorhexidine has a bactericidal effect due to cytoplasmic precipitation and/or coagulation, probably caused by protein cross-linking. The present work confirmed the efficiency of chlorhexidine as an antimicrobial agent.[3]

EDTA is an axillary substance that has a chelating action, biocompatibility with periapical tissues and optimal cleansing abilities.[14] It is considered as non-antibiotic agent and used in endodontics to remove smear layer for improve sealing during root canal obturation.15 However recent studies by M Venkatesh and Al- Bakri in 2009 have shown antimicrobial effect of EDTA against microorganisms in biofilm.15Orstavik and Haapasalo showed that EDTA has no disinfecting

action on root canal and dentinal tubule.16 However Masillamoni et al have demonstrated the antibacterial efficacy of EDTA against Staphylococcus and Streptococcus.[16] Bilge HakenSen et al have demonstrated the antifungal activity of EDTA against Candida.[17]

Wettability has been shown to play an important role in the penetration of disinfecting solutions into small spaces of root canal system. The wettability of a solution depends on its surface tension which is defined as the force between the molecules tending to reduce the surface area of a liquid. Surface tension may be reduced by the addition of chemicals known as detergents, which destabilizes cohesive forces and facilitates the distraction of extracellular polymeric substance matrix and bacterial cell membranes. In order to extend the antibacterial capability of disinfecting agents in the endodontic treatment, detergents were introduced to facilitate the medicaments entry into places of difficult access. However, the advantage of detergent in disinfecting agent only has been verified by the direct contact of medicaments with biofilm or planktonic bacteria in vitro.[10]

Cetrimide a cationic surfactant has been used in endodontics for eradication of bacterial biofilms. It is usually been used together with other irrigants.10 Arias moliz has shown in his studies that the combination of cetrimide and CHX has exhibited high antimicrobial activity against E faecalis planktonic culture.18Cetrimide was included in this study in combination with sodium hypochlorite to assess its antimicrobial activity.

All irrigants were tested with and without mechanical agitation, with better results in the groups with agitation. It could be possible that the agitation of the substance may have improved the contact between the antimicrobial agent and the micro-organism on the biofilm. Such agitation also favoured the antimicrobial agents in liquid presentation.[11]

The result of this study indicates that 2% CHX is as effective as sodium hypochlorite as an antimicrobial irrigant. The results of our study are similar to that with the study done by Vianna et al.19 Moreover Jeansonne M et al evaluated that 2% CHX is at least as effective as 5.25% NaOCl.20 The similar results were obtained in the study by Gomes et al which showed that there is no significant difference between these two irrigating solutions.[12] However it is well known that 2% CHX is less toxic than NaOCl.[21] If antimicrobial activity was the only requirement of an endodontic irrigant, the result of the study would indicate CHX is the irrigant of choice. It is as effective as NaOCland relatively nontoxic.[19,21]

EDTA and NaOCl+ cetrimide solutions were also effective to some extent in eliminating biofilms but took more time than chlorhexidine and sodium hypochlorite.

EDTA has shown the negative culture of C albicans and Staphylococcus aureus after 60 mins. This result is similar to the study done by Branin et al who reported that EDTA is known to have activity against biofilms of gram positive bacteria such as Staphylococcus aureus.14Grawehr et al demonstrated that 17% EDTA was more effective than 0.5% NaOCl against Candida albicans and staphylococcus14 which is in contrast to our study. The reason could be that we have used 3% NaOCl which has more antibacterial and tissue dissolving property than 0.5% NaOCl. The antifungal effect of EDTA as shown by Sen et al correlates with our study.[17]

Typically, although EDTA is considered as nonantibiotic agent used remove smear layer from the root canal, recent studies by Othman and Rong et al have demonstrated antimicrobial effects of EDTA against

microorganisms in biofilms. Although the mode of action of EDTA is still not clarified but it could be due to the chelating effects of calcium and iron which may affect important metabolic pathways in bacterial cells.15 In our study EDTA has shown the antibacterial effect but it was statistically different from CHX andNaOCl. Therefore, EDTA should always be used as an adjunct with CHX or NaOClrather than using it alone.

Ohara et al determined the antibacterial effects of various endodontic irrigants against selected anaerobic bacteria showing that diluting NaOClrapidly takes away its effect. This could be reason for NaOCl+Cetrimide group to show less effect as cetrimide may dilute the NaOClsolution.[19]

The protocol used in this study is practical and easy to reproduce, allowing a primary screening to test the antimicrobial effect of substances against biofilms. This study showed that different microorganisms are susceptible to different antimicrobial agents tested and the time of exposure could be decisive in the efficacy of the substances. All microorganisms grew in contact with saline with or without agitation. (positive control).[11]

This biofilm model seems to be more realistic than the direct contact method to test antimicrobial agents, as it allows micro-organisms to grow as biofilms on the nitrate cellulose membrane, which are more resistant to therapy. Further studies should test the susceptibility of antimicrobial substances against the mixed biofilms.11

Within the limitations of this in vitro study, the biofilm model gave a simple means of determining the antimicrobial efficacy of irrigants used in root canal treatment. This methodology may be more ideal than the methods, which do not consider the micro-organism in biofilms. However, it still does not reproduce what happens clinically in the root canal. In such an environment, several mechanisms allow the growth and selection of several microorganisms, even after the treatment.[11]

CONCLUSION

Within the limitation of the study it was concluded that:

2% CHX is the most effective root canal irrigant against bacterial biofilms tested. It kills the bacteria more rapidly than NaOCl, EDTA, and NaOCl+ cetrimide. However, there was no significant difference observed in CHX group and NaOCl group.

EDTA was effective against candida albicans and staphylococcus after 60 minutes and statistically different from CHX and NaOCl. EDTA was unable to show negative culture for E. faecalis and lactobacillus.

Saline did not inhibit the growth of any tested microorganism and was statistically different from all root canal irrigants used in this study.

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