In vitro Analysis of Minimal Inhibitory Concentrations of NaOCl, CHX, MTAD, and EDTA against *Enterococcus faecalis*

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Abstract

The aim of this in vitro study was to evaluate the minimal inhibitory concentration (MICs) of 3% NaOCl, 2% CHX, MTAD, and EDTA against E. faecalis.

Certified colonies of E. faecalis (ATCC 29212) were used to determine the antimicrobial effect of 3% NaOCl, 2% CHX, MTAD, and EDTA and broth dilution method was applied to determine the MIC. In the test tubes were poured testing material and the corresponding microorganism, starting from 1ml-0.06 ml of irrigant (being halved each time).

The test tubes were incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration of irrigant that inhibited bacterial growth, based on analysis using a spectrophotometer. Analysis of variance was used to compare sensitivities to the irrigants, as well as MIC.

The antibacterial effect of 3% NaOCl, 2% CHX and MTAD in E. faecalis cultures decreased with increasing dilution of irrigators. The higher rate of absorbance, the smaller the antibacterial affect of tested substances was shown and the higher purity of percentage, the greater antibacterial effect was shown.

The results of this study indicated that MTAD had the most robust and efficient antimicrobial effects, compared with other test substances, both at full concentration and when diluted fivefold.

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Introduction

To avoid formation of periapical lesions,cleaning and shaping of root canals, combined with sealing of the entire root canal system,are needed to prevent entry of fluids that would provide nutrients for remaining bacteria within root canals. [1,2] Root canal preparation should be followed by irrigation to maximize the efficiency of endodontic irrigation at the apical terminus. [3] Endodontic irrigation is necessary for removal of debris, lubrication of dentinal walls, dissolution of organic material, and provision of antimicrobial activity.[4,5]

Chemical agents used as irrigants in endodontics include tissue-dissolving agents (e.g., sodium hypochlorite), bactericidal and bacteriostatic agents (e.g., chlorhexidine [CHX] and MTAD), and chelating agents(e.g., ethylene diamine tetra acetic acid [EDTA]).Sodium hypochlorite is an efficient organic solvent that causes dentinal degeneration through collagen dissolution; however, it cannot remove the smear layer. [6] CHX exhibits antimicrobial activity and biocompatibility, but has no tissue-dissolving capabilities. [7] EDTA at concentration of 17% removes the smear layer through effects on the inorganic component of dentin, thereby facilitating removal of infected tissue and bacteria in root canals. [8] However, it has minimal antibacterial activity and contributes to dentinal erosion.[9] Thus, a combination of NaOCl and EDTA is recommended to facilitate root canal disinfection.[4] MTAD is a combination of antibiotic (doxycycline), chelator (citric acid), and detergent (Tween-80). The citric acid chelator contributes to smear layer removal, allowing doxycycline to penetrate dentinal tubules with opened orifices due to detergent effect.[9]

Despite adequate cleaning, shaping, and

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sealing of the root canal system, its anatomic complexity enables microorganisms to remain in filled root canals. *Enterococcus faecalis* is the species most commonly associated (24%–77%) with treatment failures. [10, 11]*E. faecalis* possesses multiple characteristics that enable survival in treated canals, including intracellular drug resistance, biofilm formation ability, dentinal duct invasiveness, and long-term nutrient deficiency tolerance.[12,13] Evaluation of minimal inhibitory concentration (MIC) is an important factor that affects microbial eradication.The tube dilution method is one of the most reliable methods for determining levels of resistance to an antimicrobial agent[14]; this method reveals the MIC, which is the lowest concentration of an antimicrobial agent that inhibits the visible growth of bacteria.[15] The aim of the present study was to evaluate the MICs of 3% NaOCl, 2% CHX, MTAD, and EDTA against *E. faecalis*.

Materials and methods

Certified colonies of *E. faecalis* (ATCC 29212 OXOID, Hampshire, UK) were used to determine the antimicrobial effect of in vitro irrigators.

Standardization of microorganisms

Brain heart infusion broth (BHI-Oxoid LTD., Hampshire, UK),was inoculated with *E. faecalis* and incubated for 6–7 h at 37°C to achieve a mean optical density of 0.5 McFarland constant (equivalent to 1.5×10^8 colony-forming units/ml). Then, 1-ml aliquots of each suspension culture were transferred to the required number of sterile screw cap tubes. All procedures were performed using sterilized instruments and reagents.

Irrigants used

1. NaOCl 3% (ChlorcID, Ultradent Products, Inc. South Jordan,UT, USA)
2. CHX 2% solution (Consepsis, Ultradent Products, Inc.South Jordan, UT, USA)
3. MTAD (*Dentsply Tulsa Dental, Tulsa, OK, USA)*
4. EDTA 17% (CALASEPT EDTA, Nordiska Dental, Ängelholm, Sweden).

Determination of MIC

The MIC is a reference criterion for the susceptibility of microorganisms to irrigants and endodontic drugs. To ensure the test was highly accurate, we used the broth dilution method to determine the MIC. *E. faecalis* and respective irrigants were gradually applied to the appropriate test tubes. In the tested material and the corresponding microorganism, 1ml of irrigant was applied; 1ml of the first was transferred to the second (i.e., dilution) for 0.5ml; in the second, 0.25ml was transferred; in the third, 0.125ml was transferred; and in the fourth, 0.06ml was transferred (Figure 1).

Figure 1. Broth dilution method.

The test tubes were incubated at 37°C for 24 h.The MIC was then recorded as the lowest concentration of irrigant that inhibited bacterial growth, based on analysis using a 540 nm wavelength spectrophotometer (Smart-CCD Spectrophotometer) (Figure 2).

Figure 2. Spectrophotometer for determination of absorbance and purity.

Lower absorbance and increased purity were both regarded as indicators of reduced bacterial growth. Analysis of variance was used to compare sensitivities to the irrigants, as well as MIC.

Results

In Table 1 are shown minimal inhibition concentrations and standard deviations (SD) of tested materials according to absorbance rate. It is understood from this table that the higher rate of absorbance, the smaller the antibacterial effect of the test substances. By diluting the substances, their antimicrobial activity is also reduced.

	1ml		0.5ml		0.25 Ml		0.125 ml		0.06 Ml	
	Xbar	SD	Xbar	SD	Xbar	SD	Xbar	SD	Xbar	SD
NaOCl3% absorbance	0.197	0.021	0.183	0.017	0.217	0.003	0.646	0.011	0.888	0.007
ChX2% absorbance	0.110	0.002	0.302	0.001	0.651	0.009	0.657	0.003	0.729	0.042
MTAD absorbance	0.159	0.012	0.111	0.004	0.190	0.023	0.144	0.005	0.188	0.027
EDTA 17% absorbance	0.409	0.007	0.245	0.046	0.132	0.034	0.043	0.003	0.034	0.005

Table 1. Minimal inhibition concentrations of tested materials-absorbance rate.

	1ml		0.5 ml		0.25 ml		0.125 ml		0.06 ml	
	Xbar	SD	Xbar	SD	Xbar	SD	Xbar	SD	Xbar	SD
NaOCl3% Purity	63.81	3.23	65.85	2.69	60.73	0.45	22.45	0.55	12.37	0.58
ChX2% Purity	77.83	0.40	50.08	0.01	22.14	0.60	21.80	0.02	18.70	1.78
MTAD Purity	69.51	1.86	77.84	0.40	67.23	0.26	71.62	1.10	67.28	0.21
EDTA 17% Purity	39.65	0.05	60.03	7.18	74.17	5.88	90.41	0.07	92.50	0.06

Table 2. Minimal inhibition concentrations of tested materials-purity percentage.

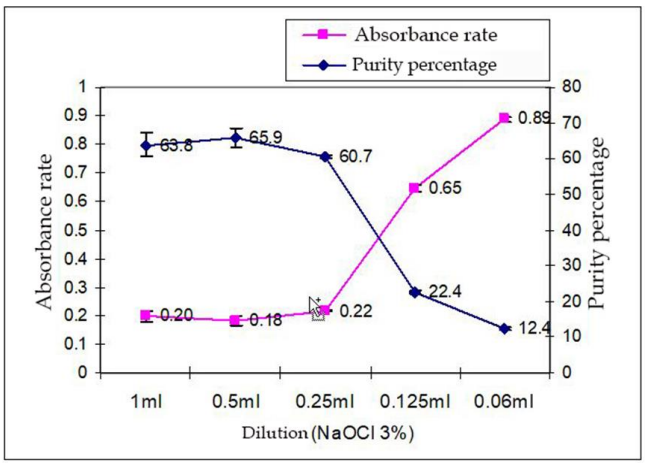

Figure 3. Minimal inhibitory concentration of NaOCl 3% against Enterococcus Faecalis.

From this table, it is understood that the higher purity percentage, the greater the antibacterial effect. By diluting the substances, their antimicrobial activity is also reduced.

The antibacterial effect of 3% NaOCl in *E. faecalis* cultures decreased with increasing dilution of 3% NaOCl: the absorbance increased from 0.2 ± 0.02 (at 1ml dilution) to 0.89 ± 0.007 (at 0.06 ml dilution). Similarly, purity decreased from 63.81 ± 3.23 (at 1ml dilution) to 12.37 ± 0.58 (at 0.06ml dilution), (Figure 3).

The antibacterial effect of 2% CHX in *E. faecalis* cultures decreased with increasing dilution of 2% CHX: the absorbance increased from 0.11 ± 0.002 (at 1ml dilution) to 0.73 ± 0.042 (at 0.06 ml dilution). Similarly, purity decreased from 77.83 ± 0.4 (at 1ml dilution) to 18.7 ± 1.78 (at 0.06ml dilution), (Figure 4).

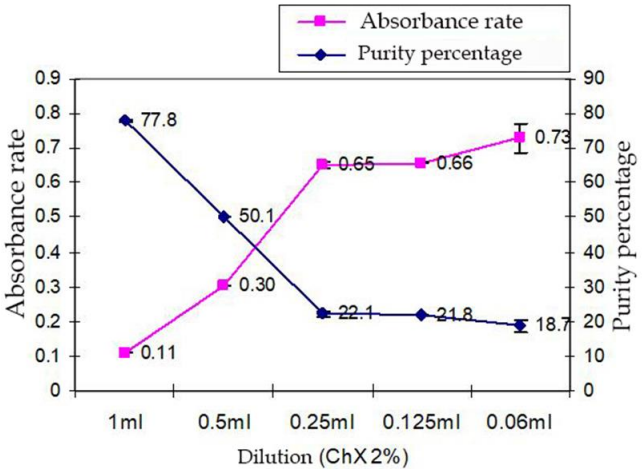

Figure 4. Minimal inhibitory concentration of 2% CHX against Enterococcus faecalis.

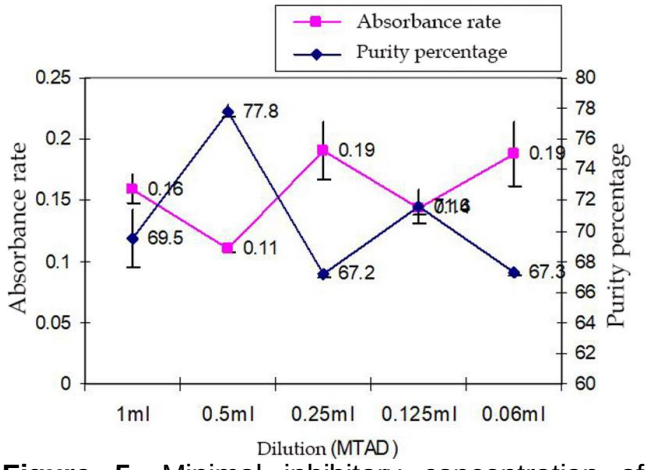

Figure 5. Minimal inhibitory concentration of MTAD against Enterococcus faecalis.

The antibacterial effect of MTAD in *E. faecalis* cultures exhibited variable outcomes,

with a general tendency to decrease with increasing dilution of MTAD:absorbance increased from 0.159 ± 0.012 (at 1ml dilution) to 0.188 ± 0.027 (at 0.06 ml dilution). Similarly, purity decreased from 69.51 ± 1.86 (at 1ml dilution) to 67.28 ± 0.21 (at 0.06ml dilution), (Figure 5).

Notably, the antibacterial effect of 17% EDTA in *E.faecalis* cultures increased with increasing dilution of 17% EDTA:absorbance decreased from 0.41 ± 0.007 (at 1ml dilution) to 0.03 ± 0.005 (at 0.06 ml dilution). Similarly, purity increased from 39.7 ± 0.05 (at 1ml dilution) to 92.5 ± 0.06 (at 0.06ml dilution), (Figure 6).

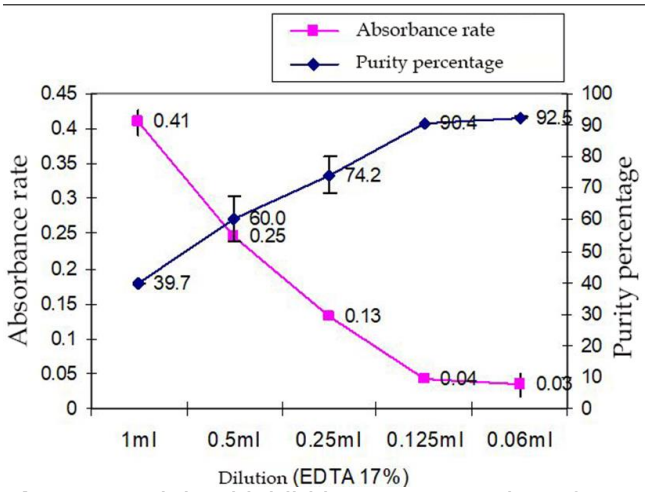

Figure 6. Minimal inhibition concentration of 17% EDTA against Enterococcus Faecalis.

Discussion

E. faecalis was selected for this study because it is the most dominant bacterial species in persistent endodontic infections. *E. faecalis* (ATCC 29212) is a commonly used quality control strain for *in vitro* studies.The tube dilution method was selected for determination of MIC because tubes can be assessed for the presence of turbidity [16]; this is one of the most basic methods for evaluation of resistance to an antimicrobial agent. [17]When the liquid remained clear after incubation of the test irrigants with*E. faecalis*, the spectrophotometer was used to determine the MIC, based on absorbance and purity.

Regarding the antimicrobial effect of the test irrigants after dilution, our study showed that some of the tested irrigants retained an antimicrobial effect after dilution, which is valuable because of their reduced toxicity. Notably, fivefold dilution of 3% NaOCl caused purity to decrease from 63.8% to 12.3%. The MIC of NaOCl for *E. faecalis* was 0.7%.These results are consistent with the findings of Heling et al. (2001), who demonstrated that the MIC for sodium hypochlorite was 0.5% for *E. faecalis.* [18]. Gomez et al. (2001) demonstrated that 0.5%, 1%, and 2.5% concentrations of NaOCl eliminated *E. faecalis* within 30 min, 20 min, and 10 min.[19] The in vitro study by Haapasalo et al. (2010) revealed that 1% NaOCl eliminated *E. faecalis* after direct contact for 5 min. [20] Conversely, Vianna et al. reported that the antibacterial activity of NaOCl was only effective at higher concentrations. [21] NaOCl and CHX are known to be effective against biofilm populations. [22, 23] Nevertheless, concentrations of NaOCl ranging from 0.00625% to 6% have demonstrated the strongest effects against*E. faecalis:*these cause bacterial lysis within 1–3 min. [22]

Our study revealed a robust antibacterial effect of MTAD up to fourfold dilution. This is consistent with the findings of prior studies. In particular, Newberry et al. (2007) showed that MTAD inhibited the growth of some *E. faecalis* strains when diluted eightfold; more over,it eliminated all strains of *E. faecalis* when diluted fivefold. [24] Tong et al. (2011) reported that the MIC of MTAD was a dilution of 8192-fold. [25] Torabinejad et al. (2003) reported that MTAD was significantly more effective in killing *E. faecalis*, compared with NaOCl, when both solutions were diluted. Measurement of MICs demonstrated that MTAD remained effective in killing *E. faecalis* at 200-fold dilution, whereas NaOCl ceased to exhibit antibacterial activity beyond 32-fold dilution. [26]

In our study, 2% CHX had a very robust antimicrobial effect against *E. faecalis*, but dilution reduced this effect. The MIC of CHX was 1%.In a previous study, Oncag et al. (2003) evaluated the antibacterial properties of 2% CHX against *E. faecalis* after 5 min and 48 h exposure. CHX exhibits a bacteriostatic effect at low concentrations and a bactericidal effect at high concentrations; moreover, it is adsorbed by tooth tissue and mucous membranes, which explains its prolonged therapeutic effect. [27] Gomes et al. (2001) and Viana et al.(2004) observed that CHX gels were significantly less effective at lower concentrations (1% and 0.2%);these produced complete bacterial growth inhibition after 15 min and 2 h, respectively. In contrast, CHX liquid at

lower concentrations (1% and 0.2%) exerted antibacterial activity against *E. faecalis* within a similar duration of time as that demonstrated by 2% CHX liquid (15 to <30 sec). [19,21] CHX at 2% produced bacterial growth inhibition within 5 min, whereas CHX at 0.2% only reduced growth to 10^7colony-forming units/ml after 1 h. [22,23]

Unlike other irrigants in our study, the antibacterial effect of EDTA on *E. faecalis* increased with dilution of EDTA;specifically, purity increased from 40% to 92% after fivefold dilution(0.06ml). This finding indicates that EDTA has stronger antibacterial effects after dilution.Fidalgo et al. (2010) showed that 17% EDTA had a stronger antimicrobial effect on *E. faecalis*, compared with 10% citric acid. [28] Similarly, Bulacio et al. (2006) reported that a 17% EDTA solution reduced the number of colony-forming units of *E. faecalis* by more than 3 log, compared with the control condition. [29] Conversely, Torabinejad et al. (2003) reported that EDTA did not exhibit any antibacterial activity. [26]

In vivo condition, E. faecalis may cause periapical disease or a failure in the root canal treatment and its virulence factor may enable this bacteria to survive in extreme environments, such as high temperature, high pH levels and high salt concentrations. [30-32]

The limitation of this in vitro study is that the findings may not be representative of in vivo outcomes; therefore, additional in vitro and in vivo studies are needed to confirm the apparent antibacterial activities and MICs of these irrigants against *E. faecalis*, to support their clinical applications.

Conclusions

The results of this study indicated that MTAD had the most robust and efficient antimicrobial effects, compared with other test substances, both at full concentration and when diluted fivefold. Furthermore, 3% NaOCl exhibited robust antibacterial effects, both at full strength and when diluted to 0.7%. Finally, 17% EDTA and 2% CHX exhibited the most robust antimicrobial effects when diluted fivefold.

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Declaration of Interest

The authors report no conflict of interest.

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