Combined effect of a mixture of tetracycline, acid, and detergent, and Nisin against *Enterococcus faecalis* and *Actinomyces viscosus* biofilms

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ABSTRACT

الأهداف: تقييم التأثير المشترك لكل من خليط من التتراسيكلين، والحامض، والمنظفات (MTAD) والنيسين (Nisin) ضد المكورات المعوية البرازية والشعيّة اللزجة في الأغشية الحيوية.

الطريقة: أُجريت هذه الدراسة خلال الفترة من شهر يونيو إلى ديسمبر 2013م وذلك بالتعاون مع كُرسي أبحاث تسوس الأسنان التابع لكلية طب الأسنان، جامعة الملك سعود، الرياض، المملكة العربية السعودية. لقد تم تكوين طبقة الأغشية الحيوية لكل نوع من البكتيريا باستخدام أقراص تصفية الغشاء (9 من الأغشية لكل نوع من البكتريا ولكل فترة حضانة). بعد ذلك تم تعريضها لحوالي 5، 10 دقائق أو 15 دقيقة حضانة مع MTADN (MTAD مع 3% من النيسين)، و 5.25% من هيبوكلوريت الصوديوم (NaOCl)، أو المحلول الملحي. بعدها تم إحصاء الوحدات المكونة للمستعمرة باستخدام مستعمرة العداد (Dark field).

النتائج: وصل التأثيرالمبيد للبكتيريا مع 5.25% من هيبوكلوريت الصوديوم خلال فترات المراقبة الثلاثة إلى 100%. فيما لوحظ انخفاض كبير (p=0.000) في معدلات البقاء على قيد الحياة بين المكورات المعوية البرازية (77.3+13.6) والشعيّة اللزجة (39.6+12.6) بعد 5 دقائق من خلطها مع MTADN وذلك بالمقارنة بالمحلول الملحي (78000000+5291503) مع انعدام النمو بعد 10 دقائق و 15 دقيقة. ولم تختلف معدلات البقاء على قيد الحياة بين المكورات المعوية البرازية والشعيّة اللزجة بعد خلطها مع MTADN و 5.25% من هيبوكلوريت الصوديوم وذلك في فترات المراقبة الثلاثة (p=1.000).

الخاتمة: لقد كانت فعالية مزج MTAD مع النيسين مماثلة لفعالية هيبوكلوريت الصوديوم وذلك ضد المكورات المعوية البرازية والشعيّة اللزجة في الأغشية الحيوية.

Objectives: To evaluate the combined effect of a mixture of tetracycline, acid, and detergent (MTAD) and Nisin against *Enterococcus faecalis (E. faecalis)* and *Actinomyces viscosus (A. viscosus)* biofilms.

Methods: This study was conducted between June and December 2013 in collaboration with Dental Caries Research Chair, College of Dentistry, King Saud University, Riyadh, Saudi Arabia. Single-species biofilms (n=9/species/observation period) were generated on membrane filter discs and subjected to 5, 10, or 15 minute incubation with MTADN (MTAD with 3% Nisin), 5.25% sodium hypochlorite (NaOCl), or normal saline. The colony forming units were counted using the Dark field colony counter.

Results: A 100% bactericidal effect of 5.25% NaOCl was noted during the 3 observation periods; a significant reduction (p=0.000) in mean survival rates of *E. faecalis* (77.3+13.6) and *A. viscosus* (39.6+12.6) was noted after 5 minutes exposure to MTADN compared with normal saline (78000000+5291503) declining to almost no growth after 10 and 15 minutes. The survival rates of the *E. faecalis* and *A. viscosus* biofilm were no different after treatment with MTADN and 5.25% NaOCl at the 3 observation periods (p=1.000).

Conclusion: A combination of MTAD and Nisin was as effective as NaOCl against *E. faecalis* and *A. viscosus* biofilms.

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Infected root canals have a complex microbial flora that may exist as a loose collection in mist canal lumen or as dense aggregates (biofilms) adhering to the dentinal walls.[1] The biofilms offer a conducive environment for bacterial growth and survival resulting in persistent periapical infections leading to potential therapeutic failure.[2,3] To avoid unfavorable outcomes due to the presence of residual microbes, after cleaning and shaping, several chemical irrigants have been applied in the root canals. Because of its potent antimicrobial activity against both planktonic and biofilm bacteria, sodium hypochlorite (NaOCl) has been used conventionally for decades as an irrigating solution.[4,5] Despite its proven efficacy as an irrigating agent in recommended concentrations, the toxic effects of NaOCl on vital tissues have been a major concern.[6]

For removal of smear layers during root canal treatment a mixture of tetracycline, acid, and a detergent (MTAD) comprising of an aqueous solution containing 3% doxycycline, 4.25% citric acid, and 0.5% polysorbate (80 detergent) with minimal erosive effects on dentin surfaces have also been used as a final rinse.[7,8] The antimicrobial effect of MTAD is primarily believed to be due to doxycycline and resistance to doxycycline is not uncommon among the bacteria isolated from the root canals.[9] The lower potency of MTAD compared with NaOCl against biofilm bacteria observed in clinical practice could possibly be due to resistance against the doxycycline component of MTAD.[4] Nisin, an antimicrobial peptide produced by *Lactococcus Lactis*, used extensively as a preservative in dairy products is composed of 34 amino acid residues, including unusual amino acids such as Lanthionine and B-methyl-lanthionine.[10,11] It inhibits proliferation of most gram-positive bacteria, is heat-stable, odorless, colorless, tasteless, non-toxic peptide and is considered safe by the U.S. Food and Drugs Administration.[12,13] Nisin when used in conjunction with MTAD has been shown to induce a significant inhibitory effect against *Enterococcus faecalis (E. faecalis)*[14,15] and some Gram-positive bacteria associated with persistent intracanal infections.[16,17] Data regarding assessment of efficacy of the combined use of MTAD and Nisin against biofilm bacteria is limited, and this study was performed to evaluate in vitro the bactericidal effect of the combined use of MTAD and Nisin against *E. faecalis* and *Actinomyces viscosus (A. viscosus)* biofilm bacteria.

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Methods. This study was conducted between June and December, 2013 in collaboration with Dental Caries Research Chair, College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

Preparation of MTAD. The MTAD (BioPure MTAD, Tulsa, OK, USA) is available as a 2-part liquid and powder product. A pre-filled 5 ml syringe (liquid) paired with the pre-filled bottle containing 150 mg of doxycycline (powder) were mixed according to the manufactures instructions. Three percent Nisin (a commercial preparation, 1000 IU/mg; Sigma Chemical Company, St. Louis, MO, USA) was added to the MTAD, mixed thoroughly in 15 ml centrifuge tubes and referred to as MTADN.

Preparation of bacterial cultures. The cultures of *E. faecalis* (ATCC 29212) and *A. viscosus* (ATCC 15987) were obtained from frozen stock culture, inoculated individually in Brain Heart Infusion (BHI) broth and allowed to grow overnight at 37°C. Cells were collected by centrifugation (1000 x g for 10 min) and the pellets were re-suspended in fresh BHI broth separately. Cultures were further diluted to obtain a McFarland unit of 0.5.

Preparation of biofilms. The biofilm model used in this study was adopted from Spratt et al.[18] Nitrocellulose membranes with 13 mm diameter (Membrane Solutions, Lane Plano, TX, USA) were sterilized in an autoclave and kept at room temperature until used. The BHI agar plates were prepared, and the filter membranes (n=9/species/observation period) were overlaid on the agar surface. Ten micro litters of the bacterial culture were dispensed on each membrane, and the plates were incubated for 48 hours under anaerobic conditions. Following incubation, individual membranes were removed aseptically from the agar plate and transferred carefully into MTADN solutions and were incubated for 5, 10, and 15 minutes at 20°C. Sodium hypochlorite (5.25%) was used as a positive control, whereas physiological saline was used as a negative control.

Enumeration of colony forming units. The membrane filters were carefully transferred to neutralizing broth containing 0.5% sodium thiosulfate and 1% glucose, and further dilutions were made in physiological saline. These log dilutions were inoculated in BHI agar plates by using a spreader, and the plates were incubated under anaerobic conditions for 3 days at the end of which time the colonies were counted using the Dark field Colony Counter (New Brunswick Scientific, Enfield, CT, USA).

Statistical analysis. Mean values of $\log_{10}$ colony forming units (CFU)/ml and standard deviations were calculated for each irrigant. The data were analyzed by using one-way analysis of variance to determine if there were significant differences in biofilm eradication among the groups at each time interval. Differences between pairs of groups were determined using the Tukey post hoc test. A p-value of either equal to less than 0.05 was considered significant.

Results. Table 1 shows data for growth inhibition of *E. faecalis* and *A. viscosus* after 5, 10, and 15 min exposure to MTADN. Compared with the negative control where a gradual decline in the mean bacterial counts was observed between 5 and 15 mins, a highly significant reduction (p=0.000) in mean bacterial counts was observed for *E. faecalis* (77.3+13.6) and *A. viscosus* (39.6+12.6) after 5 min exposure to MTADN approaching to almost no growth after 10 min and 15 min exposure. The NaOCl serving as a positive control, effectively inhibited bacterial growth of both the bacterial strains with no viable bacteria detected, at any observational time point. No significant difference (p=1.000) was observed in survival rates of *E. faecalis* and *A. viscosus* when the inhibitory effects of MTADN and 5.25% NaOCl were compared for the 3 observational time points.

Discussion. Our findings demonstrate that the combined effect of MTADN against *E. faecalis* and *A. viscosus* were similar to 5.25% NaOCl. Both the organisms have been implicated in persistent endodontic and periapical infections[19,20] and are associated with biofilm formation, which is considered as the main virulence determinant.[2] Biofilm bacteria have been shown to be 1,000 times more resistant than planktonic bacteria to phagocytosis, antibodies, antibiotics, disinfectants, and antimicrobial agents.[21] Effective eradication of primary and secondary endodontic infections particularly infections caused by biofilm bacteria is a prerequisite for a favorable outcome. The antibacterial activity of MTADN observed in this study appears to be promising specifically in the backdrop of the toxicity associated with NaOCl.

The efficacy of the combined use of Nisin and MTAD in the present study was evident, and has also been reported previously.[14-16] Nisin, a cationic peptide, appears to be critical for the enhanced antibacterial activity, it has been shown to dissipate the membrane potential and the pH gradient in liposomes leading to inhibition of oxygen consumption by cytochrome oxidases and eventually killing the bacteria.[11] In addition, Nisin is also capable of effectively inhibiting the growth and biofilm formation by *S. aureus*,[17] *E. faecalis*, and *S. gordonii*.[22] Moreover, a study comparing the bactericidal effect of MTADN and MTAD on 10 different isolates of *E. faecalis* has recently demonstrated that performance of MTADN was significantly better than MTAD.[15] Similarly when compared with MTAD, MTADN has also been shown to perform better as an antibacterial agent against common pathogens associated with root canal infection including *A. viscosus*,[16] which was consistent with the findings of the present study. Actinomyces species is well known for their ability to form oral biofilms,[23] and have been implicated in treatment failures, including those associated with extra-radicular infections.[24] Furthermore, the antibacterial effect of MTADN against *Actinomyces naeslundii* has been attributed to MTADN mediated cell rupture.[16]

The fact that there was no significant difference in the survival rates of the *E. faecalis* and *A. viscosus* biofilm after exposure to MTADN at the 3 observation periods indicates that the antibacterial effect of MTADN is not selective. This finding is consistent with other reports demonstrating the inhibitory effect of MTADN against *E. faecalis*, and some gram-positive bacteria frequently implicated in persistent intracanal infections.[14,16] The proposed mechanism of the antibacterial effect of MTADN is believed to be the induction of pores by Nisin on the surface of the cell membrane that may facilitate the penetration of doxycycline molecules into the microorganisms.[10] The combined use of Nisin with MTAD as an effective bactericidal agent observed in the

Table 1 - Susceptibility of *Enterococcus faecalis (E. faecalis)* and *Actinomyces viscosus (A. viscosus)* to MTADN, NaOCl, and normal saline.

Time (mins)	Number of viable bacteria (mean ± SD) *E. faecalis*			Number of viable bacteria (mean ± SD) *A. viscosus*		
	Normal saline	MTADN	NaOCl	Normal saline	MTADN	NaOCl
05	78000000 ± 5291503	77.3 ± 13.6*	0	7000000 ± 984885.8	39.6 ± 12.6*	0
10	58333333 ± 26633312	0	0	5700000 ± 1400000	3 ± 1*	0
15	40666667 ± 14224392	0	0	4933333 ± 873689.5	0	0

MTADN - mixture of tetracycline, acid, detergent and Nisin, NaOCl - sodium hypochlorite, *$p \leq 0.001$ statistically significant

present study highlights the considerable potential of MTADN to be used as an effective irrigating solution.

The biofilm model used in this study is useful as a rapid primary screen to test the antimicrobial effect against biofilms. Growing biofilms on standardized readily available surfaces allows a more accurate assessment of antimicrobial efficacy and has the advantage of testing a large number of variables rapidly with a relative ease. This model may also obviate the need for an extracted tooth and the time-consuming preparation. However, this model does not account for the anatomical variations in individual teeth. Although single bacterial specie may not be representative of the polymicrobial infection in the root canal, this could however simplify the biofilm formation as the multispecies biofilms have different modes of growth, which is difficult to control and represents a problem in disruption assessment.[25] The exposure time of the tested irrigants was selected based on the minimum and maximum time expected for cleaning and shaping with the current rotary instruments. Lack of significant difference in biofilm eradication of MTADN at the 3 exposure periods indicates that a 5 min contact time may be considered optimal as a final rinse to comply with the recommendation of Torabinejad et al.[8] The in vitro performance of MTADN in the present study as an effective bactericidal agent against *E. faecalis* and *A. viscosus* emphasizes the need for further assessment of MTADN as an alternative and a relatively less toxic irrigant for endodontic treatment.

In conclusion, based on our study results the MTAD when used in combined with Nisin appears to be an effective and a potent intracanal irrigating solution.

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