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REMOVAL OF A DUAL-SPECIES BIOFILM FROM ARTIFICIAL LATERAL CANALS

Abstract

Lateral canals are particularly challenging to clean and disinfect. The aim of this study was to compare the removal efficacy of a dual-species biofilm from a lateral canal model by different ultrasonic irrigant activation protocols *in vitro*. Aim is quantify biofilm removal from a simulated isthmus and a lateral canal in an artificial root canal system during syringe irrigation with NaOCl at different concentrations and delivered at various flow rates b. to examine whether biofilm removal is further improved by a final high-flow-rate rinse with an inert irrigant following irrigation with NaOCl.

Keywords: *biofilm, lateral canal, optical coherence tomography, ultrasonic irrigant activation, in vitro*

Qələndər Xanlar oğlu Əliyev

İkili növlü biofilmin süni laterial kanallardan çıxarılması

Xülasə

Yan kanalları təmizləmək və dezinfeksiya etmək xüsusilə çətindir. Bu tədqiqatın məqsədi iki növlü biofilmin müxtəlif ultrasəs irriqant aktivləşdirmə protokolları ilə laterial kanal modelindən çıxarılmasının effektivliyini *in vitro* şəraitində müqayisə etməkdir. Məqsəd müxtəlif konsentrasiyalarda və müxtəlif axın sürətlərində NaOCl ilə şprislə yuma zamanı laterial kök kanalı sistemində simulyasiya edilmiş yan kanaldan biofilmin çıxarılmasının kəmiyyətini müəyyən etməkdir. NaOCl ilə yuduqdan sonra inert irriqantla son yüksək axınlı yaxalama ilə biofilmin çıxarılmasının daha da yaxşılaşdırılıb-yaxşılaşmadığını yoxlamaq.

Açar sözlər: *biofilm, laterial kanal, optik tomoqrafiya, ultrasəs irriqant aktivləşdirmə, in vitro*

Introduction

Methods

Artificial root canal models with 270 simulated lateral canals were made of polydimethylsiloxane. A dual-species biofilm (*Streptococcus oralis* and *Actinomyces naeslundi*) was grown *in vitro* in the lateral canals using a constant depth film fermenter. Two percent NaOCl or demineralized water was delivered by a syringe and an open-ended needle for 30 seconds and subsequently activated by an ultrasonic file for a total activation time of 30, 60, or 90 seconds divided in 1 or 3 consecutive activation cycles. In the control groups, the irrigant was allowed to rest for 30, 60, or 90 seconds. The volume of the biofilm in the lateral canal was evaluated before and after the final irrigation protocol by optical coherence tomography. The results were analyzed by 3-way factorial analysis of variance ($\alpha = 0.05$).

Irrigation with NaOCl rather than demineralized water resulted in more effective biofilm removal from the lateral canal ($P < .001$). Three cycles of intermittent ultrasonic activation were significantly more effective than no activation ($P = .029$). The total irrigant contact time did not affect biofilm removal ($P = .403$).

The type of the irrigant and the ultrasonic activation protocol affected biofilm removal from artificial lateral canals. None of the compared protocols was able to eradicate the biofilm.

Elimination of bacteria from an infected root canal system is one of the main goals of root canal treatment. Irrigation with antimicrobial solutions is currently considered the primary means of root canal disinfection. Irrigants are most often delivered by a syringe and a needle, but this technique is unable to create an adequate flow inside narrow areas such as lateral canals and isthmuses (Verhaagen, Boutsioukis, Sleutel, 2013: 1165-1177; Boutsioukis, 2019: 303-321).

As a result, a considerable amount of intact biofilm may remain there after chemomechanical preparation (Nair, 2004: 348-381; Nair, Henry, Cano, Vera, 2005: 231-252). Persistent biofilm infection in lateral canals has been implicated in root canal treatment failure (Ricucci, Loghin, Sigueira, 2013: 712-718).

Numerous irrigant agitation and activation methods have been proposed in order to improve the cleaning and disinfection of canal ramifications, ultrasonic activation being the most popular one (Dutner, Mines, Anderson, 2012: 37-40). A wide variety of ultrasonic activation protocols are currently in use without any consensus on the most effective one (Căpută, Retsas, Kuijk, 2019: 31-44). Repeated activation of NaOCl for short periods leads to more effective removal of dentin debris from artificial grooves *in vitro* than a single long activation period (Van der Sluis, Gambarini, 2006: 472-476). However, the removal of dentin debris by activated NaOCl is likely to be a predominantly mechanical process, so the consumption of the free available chlorine, which may affect biofilm removal (Arias-Moliz, Morago, Ordinola-Zapata, 2016: 771-775), was not taken into account. Moreover, a viscoelastic biofilm firmly attached to dentin (Busanello, Petridis, So, 2019: 461-474) may react differently to the oscillating pressure and shear stress during activation (Verhaagen, Boutsioukis, Sluis, Versluis, 2017: 1717-1730) compared to the more rigid but less firmly attached dentin debris. Thus, the activation protocols that promote the removal of dentin debris may not be as effective against biofilm.

A dense dual-species biofilm (*Streptococcus oralis* and *Actinomyces naeslundii*) grown in transparent artificial isthmuses and lateral canals using a constant depth film fermenter (CDFF) has been previously used to evaluate the performance of various irrigants and irrigation methods *in vitro (*Pereira, Dijkstra, Petridis, 2021: 112-129; Pereira, Boutsioukis, Dijkstra, 2021: 427-438; He, Peterson, Jongsma, 2013: 93750). This biofilm has realistic viscoelastic properties (Riccuci, Siqueira, 2010: 1-15) and resembles naturally cell-rich root canal biofilm formed under space limitations (Busanello, Petridis, So, 2019: 461-474). In addition, neither syringe irrigation nor ultrasonic activation is able to eliminate this biofilm from artificial lateral canals (Pereira, Boutsioukis, Dijkstra, 2021: 427-438) so it could serve as an adequate challenge when comparing different ultrasonic activation protocols. *In situ* three-dimensional evaluation of the biofilm volume before and after irrigation using optical coherence tomography (OCT) could account for the inevitable variability in its volume even when it is grown *in vitro* under strictly controlled conditions (Busanello, Petridis, So, 2019: 461-474; Petridis, Busanello, So, 2019: 1773-1788).

Therefore, the aim of this study was to compare the removal efficacy of a dual-species biofilm from an artificial lateral canal model by NaOCl or demineralized water activated ultrasonically according to different protocols.

A sample size estimation was conducted *a priori* using G∗Power 3.1.9 (24), assuming a three-way factorial analysis of variance ($f = 0.25$; $\alpha = 0.05$; power = 90%). The calculated minimum sample size of 14 biofilm specimens per group was increased to 15 in order to compensate for any uncertainty in these assumptions.

Artificial Root Canal Systems

Fifty-four transparent artificial root canals (apical size 35/.06 taper, 18-mm long, apically closed system) with suitable cylindrical recesses were fabricated from polydimethylsiloxane (PDMS; Sylgard 184; Dow-Corning, Midland, MI, USA) as described previously (He, Peterson, Jongsma, 2013: 63750). Cylindrical molds with thin metal pins were used to create 270 PDMS inserts with simulated closed-ended lateral canals ($d = 0.25$ mm, $l = 1.5$ mm, volume = 0.075 mm^3) that fitted the cylindrical recess of the root canal models. Degassed PDMS was poured into the molds and left to cure for 24 hours at room temperature in order to avoid bubble entrapment. When the models were assembled, the entrance of the lateral canal was located 2 mm from the apical endpoint of the main root canal.

Iofilm Formation−A steady-state dense cell-rich dual-species biofilm was grown in the artificial lateral canals using a CDFF, similarly to earlier studies (He, Peterson, Jongsma, 2013: 63750). A single colony

of either *S. oralis* J22 or *A. naeslundi* T14V-J1 was used to inoculate 10 mL of modified brain heart infusion broth (BHI; Oxoid Ltd, Basingstoke, UK), which was cultured at 37°C for 24 hours in ambient air (*S. oralis*) or in anaerobic conditions (*A. naeslundii*). These precultures were used to inoculate 200 mL of modified BHI, which was incubated at 37°C for 16 hours. The bacteria were harvested by centrifugation (6500 × g) and washed twice in sterile adhesion buffer (0.147 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, 0.136 g/L KH_2PO_4, 3.728 g/L KCl, pH 6.8). The suspension was sonicated intermittently in ice water (3 × 10 s) to break the bacterial chains. The bacteria were then counted in a Bürker-Türk chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) and diluted in sterile adhesion buffer.

Stimulated human whole saliva was collected from volunteers in accordance with the guidelines of the Institutional Ethics Committee (approval letter 06-02-2009). The collected saliva was pooled, centrifuged to remove food particles and cells, stabilized by adding a protease blocker, and freeze-dried for storage. Immediately before use, freeze-dried saliva was dissolved in adhesion buffer (1.5 g/L), stirred for 2 hours, and centrifuged (10,000 × g at 10°C) for 5 minutes in order to create reconstituted human whole saliva (RWS). RWS was pipetted on each PDMS insert and left undisturbed for 14 hours at room temperature.

The CDFF along with the inserts and culture media was autoclaved for 30 minutes at 121° C. Subsequently, the RWS-coated PDMS inserts were loaded on the turntable of the CDFF, and dropwise inoculation with 100 mL of the dual-species bacterial suspension (*S. oralis* 6×10^8 bacteria/mL and *A. naeslundii* 2×10^8 bacteria/mL) took place over 1 hour while the turntable rotated slowly. Next, rotation was halted, and the bacteria were allowed to adhere to the inserts for 30 minutes. Rotation was then resumed, and modified BHI was continuously supplied (45 mL/h) to allow the biofilms to develop during the next 96 hours at 37°C. The fixed scraper blades of the CDFF applied the necessary pressure for the mechanical compression of the biofilm and also distributed nutrients over the inserts.

The biofilm-filled inserts were stored inside a jar containing adhesion buffer until use to prevent dehydration and were only fitted in the cylindrical recess of the artificial root canal immediately before the experiments. A fluid-tight fit was ensured.

Optical Coherence Tomography

The biofilm was scanned in three dimensions by a spectral-domain OCT scanner (Ganymede II; Thorlabs, Newton, NJ, USA) with a 930 nm center wavelength white light beam using a 5 × 5 × 1.34 mm field of view (1000 × 1000 × 500 pixels) and a refractive index of 1.33 for the biofilm. The imaging frequency was 30 kHz with a sensitivity of 101 dB. The scans were initially processed with ThorImage OCT 5.5 software (Thorlabs) and then exported to Fiji 1.50g as 8 bit images in order to calculate the volume of the lateral canal that was occupied by biofilm following automatic observer-independent segmentation. No attempt was made to distinguish between the bacteria and the extracellular polymeric substance.

Statistical Analysis

The difference in the biofilm volume before and after irrigation was divided with the initial volume to calculate the percentage of the biofilm removed from each lateral canal. The effect of the type of irrigant, the number of activation cycles, and the total contact time with the irrigant was analyzed by 3-way factorial analysis of variance. Normality was evaluated in Q-Q plots, and equality of error variances was assessed by Levene's test. The null hypothesis was that the type of irrigant, the number of activation cycles, and the total contact time with the irrigant have no significant effect on the percentage of the biofilm that was removed.

Discussion

Lateral canals in the middle and apical third of the root are particularly challenging to clean and disinfect during nonsurgical treatment so irrigant activation methods that are able to reach these areas are indispensable (20). The aim of this study was to determine the optimum ultrasonic activation protocol for biofilm removal from a lateral canal *in vitro*, a topic that had not been addressed in the literature. The findings reaffirmed the importance of NaOCl and multiple activation cycles for this process.

A large part of the observed biofilm removal was attributed to the disruptive chemical effect of NaOCl (Macedo, Wesselink, Zaccheo, 2010: 1108-1115), which appeared to be more important than the pure mechanical cleaning effect of ultrasonic activation that was demonstrated in the groups irrigated with demineralized water. However, it is likely that ultrasonic activation was also able to enhance the chemical

effect of NaOCl (Malentacca, Uccioli, Zangari, 2012: 1622-1626). Agitation of the NaOCl in the main canal could have maintained a favorable concentration gradient driving the diffusion of molecules and ions into the lateral canal, where they could react with the biofilm.

Previous studies have shown that ultrasonic irrigant activation may improve the removal of pulp tissue remnants, dentin debris, or biofilm from lateral canals or other artificial ramifications *in vitro* (Pereira, Boutsioukis, Dijkstra, 2021: 427-438). A significant difference in biofilm removal from a lateral canal was found in the present study when comparing 3 activation cycles to no activation, while no difference was found between a single activation cycle and no activation, when the total contact time with the irrigant was held constant. These findings were in agreement with earlier reports on the removal of dentin debris. The main advantage of intermittent activation for short periods over continuous activation for a longer period has been attributed to the intense streaming produced during the start-up phase (first 50 ms) of activation (Jiang, Verhaagen, Versluis, 2010: 1887-1891).

The total irrigant contact time did not affect biofilm removal in this model. Since the contact time plays a critical role in both the diffusion (Verhaagen, Boutsioukis, Sleutel, 2013: 1165-1177) and the chemical reactions taking place, this finding appeared to contradict the importance of the chemical effect of NaOCl. It could be hypothesized that the contact times examined in the present study (60–120 s) were not long enough to have a noticeable effect on the diffusion or the chemical reactions, given the limited contact surface between the irrigant and the biofilm in the lateral canal. A previous study indicated that the chemical disruption of biofilm *in vitro* by NaOCl diffusing across a small contact surface became noticeable only after an extended time (300 s) (Petridis, Busanello, So, 2019: 1773-1788). Longer contact times should be tested in future studies, but it should be noted that prolonged activation may also lead to inadvertent removal of more dentin.

Under ideal conditions, irrigant flow can penetrate the lateral canal up to a distance of twice its diameter before diffusion becomes the dominant transport process (Verhaagen, Boutsioukis, Sleutel, 2013: 1165-1177). This distance corresponds to one third of the length of the lateral canal used in the present study, but the biofilm was not consistently removed from this area. It is likely that the biofilm created an additional physical obstacle for the flow that was not taken into account in the earlier calculations (Verhaagen, Boutsioukis, Sleutel, 2013: 1165-1177). Moreover, even though the irrigant flow pattern inside empty lateral canals seems to correlate well with biofilm removal *in vitro* (He, Peterson, Jongsma, 2013: 63750), mere penetration of the irrigant may not be enough to achieve total elimination of the biofilm.

The current *in vitro* model has some limitations that should be taken into account. The artificial root canals were made of PDMS to ensure dimensional accuracy (He, Peterson, Jongsma, 2013: 63750) and allow high-resolution measurements of the biofilm volume by OCT. Even though biofilm can adhere to PDMS and resist removal by a variety of irrigation methods it remains unclear whether the adhesion to PDMS is similar to the adhesion of real root canal biofilm on dentin *in vivo*. Due to technical difficulties, the length and diameter of the simulated lateral canals were within the ranges of real lateral canals but close to their upper limits. The conditions may be slightly different in smaller canals. It should also be emphasized that the chemical effect of NaOCl may have been overestimated due to the absence of dentin and accumulated dentin debris that could have partially consumed the available chlorine or impeded the access of the irrigants to the biofilm (Malki, Verhaagen, Jiang, 2012: 657-661). Ethylenediaminetetraacetic acid was not used in the experiments in order to reduce the number of confounders, even though it can destabilize the biofilm matrix (Petridis, Busanello, So, 2019: 1773-1788), so it could have facilitated biofilm removal during subsequent irrigation with NaOCl.

OCT is a noninvasive method to quantify biofilm removal in three dimensions that allows for repeated measurements on the same specimens before and after irrigation in order to account for the inevitable variation in the initial biofilm volume. However, it cannot distinguish between alive and dead bacteria or between bacteria and extracellular polymeric substance. Confocal laser scanning microscopy combined with fluorescent staining of the biofilm could have provided such information about the biofilm remaining after irrigation (Verhaagen, Boutsioukis, Van der Sluis, 2014: 1717-1730), but it would not have been able to determine the initial condition of the biofilm in each specimen before irrigation. Previous studies have validated the use of OCT for the assessment of a similar dual-species cell-rich biofilm by comparison

to confocal laser scanning microscopy (Petridis, Busanello, So, 2019: 1773-1788). Biofilm removal rather than bacterial killing has also been suggested as the preferable outcome in root canal treatment because various bacterial components can still induce periapical inflammation even after bacteria are killed (Faul, Erdfelder, Lang, Buchner, 2007: 175-193).

Conclusions

Irrigation with NaOCl rather than demineralized water and 3 cycles of intermittent ultrasonic activation rather than no activation resulted in significantly more biofilm being removed from the simulated lateral canal. The total irrigant contact time did not affect biofilm removal. None of the tested protocols was able to eradicate the biofilm.

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