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Candida albicans Antimicrobial and Antibiofilm Activity of Novel Endodontic Solvents

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Abstract: Background: *Candida albicans* is the most prevalent fungi isolated in endodontic infections. In this study, the ability of *C. albicans* biofilm to tolerate the novel solvent mixtures methyl ethyl ketone (MEK)/tetrachloroethylene (TCE) and MEK/orange oil (OOil) sequentially to the standard irrigation of sodium hypochlorite (NaOCl) and ethylenediaminetetraacetic (EDTA) was evaluated. Methods: Biofilm cell cultures of *C. albicans* SC 5314 were treated sequentially with NaOCl and EDTA and exposed to MEK/TCE or MEK/OOil. The effect of the antimicrobial treatment was evaluated using the disk diffusion method for planktonic culture, and the enumeration of colony-forming units (CFUs) and scanning electron microscope (SEM) for biofilm cell culture. Results: *C. albicans* mature biofilm (24 h) was significantly reduced in biomass and cell viability after solvent mixtures' exposure, compared with the previous NaOCl and EDTA treatments. MEK/OOil combination caused a total reduction of biofilm, while with MEK/TCE, there was a 3-log (CFU/cm^2) reduction compared with the sequence NaOCl and EDTA, and a 4-log (CFU/cm^2) reduction compared with the control. Conclusions: The additional exposure of a preformed 24 h *C. albicans* biofilm to novel solvent mixtures MEK/TCE and MEK/OOil caused a positive antibiofilm impact, overcoming the performance of the conventional endodontic irrigating protocol.

Keywords: adjunctive approaches; antibiofilm; antimicrobial; *Candida albicans*; endodontic infections; endodontic solvents; endodontic retreatment; irrigating solutions; root canal infection; solvent mixtures

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1. Introduction

Apical periodontitis (AP) is a biofilm-induced disease caused by a multicellular microbial community into the root canal system [1]. The presence of signs and/or symptoms related to endodontically treated teeth is referred to as post-treatment AP, caused by persistent/secondary intraradicular infections. The involved microflora is different from primary infections, with a predominance of Gram-positive bacteria and certain fungi [2].

The growing rate of the emergence of multi-drug-resistant pathogenic fungi has also promoted the resistance of opportunistic ones worldwide [3]. Although still unclear, the precise role of fungi in root canal infections has been emphasized either in its onset or along the disease process due to a certain bacterial–fungal co-occurrence [4]. Ubiquitous human commensal fungi, as the yeast *Candida albicans*, present opportunistic features, justifying their role, both in primary AP and post-treatment disease [2]. The potential to form resilient biofilm communities, adhere to dentin and root filling materials, and evade host defenses, with special relevance in the apical third, may account for their persistence, challenging the standard endodontic procedures [2,5]. *C. albicans* has been the most frequently isolated fungi, particularly associated to persistent over primary endodontic infections [2], although a similar prevalence between them has been recently reported [4,6].

The conventional nonsurgical endodontic treatment/retreatment is the therapy of choice to face primary or post-treatment endodontic infections, respectively. Despite the fact that AP is more prone to develop in teeth with inadequate restorative and endodontic procedures [7], its prevalence continues to increase, with great focus on root canal-treated teeth (post-treatment AP) [8–10]. Retreatment's main purpose is to reduce the intraradicular microbial load to a level that enables periapical healing [11], removing the maximum of the potentially infected existing root fill. The exposure of the empty and (re)prepared root canal to the irrigating sequence of NaOCl and EDTA is crucial not only to improve disinfection but also to achieve an adequate dentin–sealer interaction for a hermetic reseal [5,12,13].

The use of the chemical irrigants sodium hypochlorite (NaOCl) and ethylenediaminetetraacetic acid (EDTA) as the recommended protocol remains unquestionable. NaOCl is universally used, with emphasis on tissue dissolution/root canal debridement and antimicrobial/antibiofilm activity [12,14–16]. Its efficacy against *Candida* cells, recognized as refractory to endodontic irrigants and dressings, was confirmed independently of the concentration of tested strains [17]. However, different *Candida* biofilm phenotypes were reported as tolerant to the standard NaOCl [5]. EDTA, indicated for smear layer removal, significantly inhibited regrowth after NaOCl exposure, although viable *Candida* cells were still observed. Furthermore, the improved delivery and efficacy of NaOCl activation by different devices [18], in addition to the reduction in unprepared root canal areas through a greater apical enlargement [19], does not necessarily correlate with superior disinfection [20–22].

Given the concern about antibiotic resistance, an emphasis on natural products derived from plants, such as essential oils, was simulated, but there are still contradictory results in the dentistry field [23,24]. A renewed approach and interest in traditional endodontic solvents did not achieve the expected outcomes in what concerns filling removal [25,26]. Regarding its antibacterial activity, apart from the proven effectiveness of the cytotoxic chloroform [27], there are few reports about the antimicrobial/antibiofilm efficacy of other endodontic solvents [12,28,29]. Recently, Ferreira et al. investigated a novel endodontic retreatment strategy, incorporating non-traditional and safer endodontic solvents, with a different rationale in its application [30–34]. Preliminary results led to the suggestion of using the binary mixtures methyl ethyl ketone/tetrachloroethylene (MEK/TCE) or methyl ethyl ketone/orange oil (MEK/OOil) as an additional and adjuvant step in filling remnants removal. We hypothesized that the unexpectedly good performance in filling dissolution, obtained through solvents association, could also be reflected in superior disinfection, per se. Therefore, the present work aimed to evaluate the antimicrobial/antibiofilm activity of the solvent mixtures MEK/TCE and MEK/OOil against *C. albicans* planktonic cells and biofilm. The null hypothesis tested was that the solvent mixtures would not have an additional effect over NaOCl and EDTA treatment.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

Candida albicans SC5314 (ATCC MYA-2876) strain was used in this study and stored at –80 ± 2 °C in broth medium with 20% (*v*/*v*) glycerol. Before each assay, the *C. albicans* strain was subcultured from the frozen stock onto Sabouraud dextrose agar (SDA) plates. SDA was prepared from Sabouraud dextrose broth (30 g/L-SDB; Liofilchem) supplemented with 2% (*w*/*v*) agar (Liofilchem). The plates were incubated aerobically at 37 °C for 18–24 h.

2.2. Test Agents

Different irrigating solutions included in the standard endodontic protocol, i.e., 3% sodium hypochlorite (NaOCl, CanalPro, Coltene Whaledent, Altstatten, Switzerland), 17% ethylenediaminetetraacetic acid (EDTA, CanalPro, Coltene Whaledent, Altstatten, Switzerland), and the solvent mixtures MEK/TCE (1:1) and MEK/OOil (1:1), suggested as an adjuvant step, were tested as antimicrobial agents. The isolated solvents methyl ethyl ketone (MEK; VWR International SAS, Paris, France), tetrachlorethylene (TCE; VWR

International SAS, Paris France), and orange oil (OOil; Citrol, Biodinamica, Brazil) were also assessed. All solvents were stored at room temperature, protected from light.

2.3. Antimicrobial Activity against Planktonic Cells

The *C. albicans* SC 5314 strain was inoculated into 20 mL of SDB from SDA plates not older than 2 days and grown for 18–24 h at 37 °C in an orbital shaker at 120 rpm. After inoculation, the culture was centrifuged (3000× *g*, 4 °C, 10 min), washed, and the pellet was then resuspended. The cell concentration was adjusted to 1 × 105 cells/mL using a Neubauer counting chamber. An aliquot of each strain (300 µL) was spread onto SDA plates. An aliquot (25 µL) of each solvent mixture under study was placed on a sterile blank disk, which was placed onto each SDA plate above the lawn of cells. Phosphate-buffered solution (PBS1x; 0.1 M, pH = 7.5) was used as a negative control. Then, plates were incubated at 37 °C for 24 h, and the inhibitory effects were determined by measuring the corresponding zones of inhibition (inhibition halo diameter). All procedures are standardized in our laboratory.

2.4. Antibiofilm Activity

Biofilms were formed based on a microtiter plate test described by Stepanovic et al. [35], with some modifications. The plates, prepared as described above, were incubated aerobically at 37 °C for 18–24 h. After inoculation, the culture was centrifuged (3000× *g*, 4 °C, 10 min) and washed, and the pellet resuspended. The cell concentration was adjusted to 1×10^8 cells/mL using a Neubauer counting chamber. For biofilm formation, 2 mL of the cell suspensions were transferred to 24-well flat tissue culture plates with steel coupons (1 cm × 1 cm). To allow for biofilm formation, the culture plates were incubated aerobically for 24 h on a horizontal shaker at 120 rpm and 37 °C. Negative controls were performed using wells containing only SDB medium.

In order to mimic clinical endodontic retreatment, biofilms were immersed in the following conventional irrigating solutions: in 3% NaOCl for 5 min followed by 17% EDTA for 1 min, and finally in the isolated solvents (MEK, TCE, OOil) and mixtures (MEK/TCE or MEK/OOil) for 5 min each. Negative controls were performed using, sequentially, 3% NaOCl for 5 min, 17% EDTA for 1 min, and phosphate-buffered saline for 5 min. We also used a control culture of *C. albicans* SC5314 without any treatment. After this procedure, the biofilm cells were scraped, and their cultivability was assessed by colony-forming unit (CFU) calculation. In brief, 1 mL of fresh saline solution was added to each well plate, and the biofilms were scraped from the steel coupons. The biofilm cells suspensions were then serially diluted in saline solution and plated onto SDA plates. After 24 h of aerobic incubation at 37 °C, the cultured cell count was performed. Values of cultivable sessile cells were expressed as Log CFU per area (Log CFU/cm^2).

The biofilm cultures, with and without treatment with the conventional irrigating sequence of NaOCl and EDTA, and the solvent mixtures were also observed by scanning electron microscopy (SEM) in an EDAX Nova nanoSEM 200 equipment. In brief, samples were carefully washed three times with distilled water, dehydrated by immersion in solutions of increasing ethanol concentrations of 70, 95, and 100% (*v*/*v*) for 10, 10, and 20 min, respectively, and placed in a sealed desiccator. Afterward, samples were mounted in aluminum pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were sputter-coated with gold and observed on a Phenom Charge Reduction Sample Holder (CRH) at 5 Kv and a spot size of 3.3, with a desktop SEM coupled with energy-dispersive X-ray spectroscopy (EDX) analysis (Phenom ProX with EDX detector, Phenom-World BV, Eindhoven Netherlands). All procedures are standardized in our laboratory.

2.5. Statistical Analysis

Data were analyzed using the Prism software package (GraphPad Software version 6.01 for Macintosh). One-way ANOVA and *t*-tests were used, considering statistical

significance at $p < 0.05$. At least three independent experiments (performed in triplicate) were carried out for all assays.

3. Results

3.1. Antimicrobial Activity

The antimicrobial activity of MEK, TCE, OOil, MEK/TCE, and MEK/OOil against *C. albicans* was assessed using the disk diffusion assay (Table 1).

Table 1. Antimicrobial activity (disk diffusion method) of different solvents against *C. albicans* SC 5314 (* means $p < 0.05$).

Solvent	Inhibition Zones (mm)
MEK	8.7 ± 1.2
TCE	9.0 ± 1.4
OOil	50.8 ± 7.2 *
MEK/TCE (1:1)	13.8 ± 1.8
MEK/OOil (1:1)	45.0 ± 5.0 *

Among the tested solvents, OOil was the most effective, with an inhibition zone of 50.8 ± 7.2 mm. On the other hand, the contact with MEK and TCE individually resulted in weaker inhibition zones. A higher inhibition halo was observed with both mixtures, particularly with MEK/OOil. These results indicate a good diffusion capacity of the compounds and a preliminary positive antifungal activity against *C. albicans*, mainly when OOil was present.

3.2. Antibiofilm Activity

The effect of the two solvent mixtures MEK/TCE and MEK/OOil was evaluated on *C. albicans* SC 5314 biofilms formed during 24 h (Figure 1) by comparison with the solvents alone and the standard protocol. Washing with NaOCl and EDTA (standard procedure in clinical practice) resulted by itself in a reduction of about 1 Log (CFU/cm^2) (Figure 1A). After contact of the biofilm with both solvent mixtures, a further significant reduction in biomass was observed. Interestingly, the MEK/OOil association caused a total elimination of the preformed biofilm. MEK/TCE achieved a 3-log reduction compared with the use of NaOCl and EDTA and a 4-log reduction compared with the control (Figure 1A). The subsequent contact of the NaOCl-EDTA-treated *C. albicans* biofilms with the isolated solvents showed different efficacies, with complete cell eradication after TCE and OOil exposures, and a significant reduction (CFU/cm^2) with MEK, showing good efficacy of these compounds by themselves.

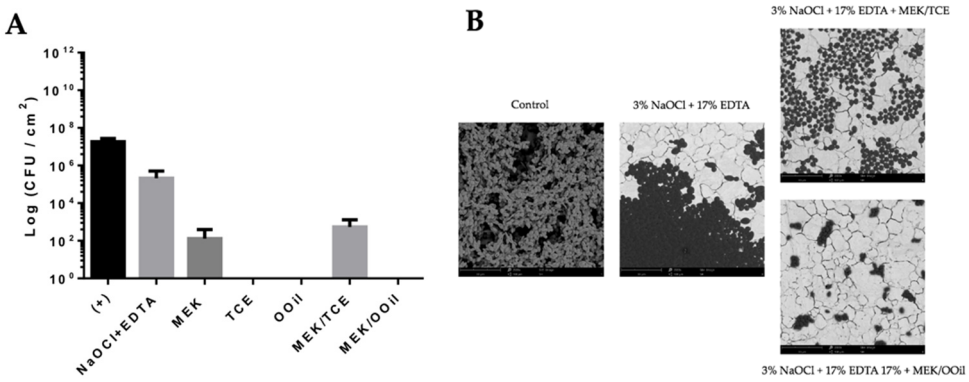

Figure 1. (**A**) Viable cell counts of *Candida albicans* SC 5314 biofilms performed after 24 h of aerobic incubation at 37 °C. Values of cultivable sessile cells were expressed as Log CFU per area (cm^2). (**B**) SEM analysis of the development of 24-h biofilms of *C. albicans* SC 5314 after exposure to the conventional final irrigation protocol (3%NaOCl + 17%EDTA) followed by the solvent mixtures (MEK/TCE or MEK/OOil).

The SEM results (Figure 1B) show the benefit of applying the suggested mixtures (MEK/TCE or MEK/OOil) after treatment with NaOCl and EDTA.

4. Discussion

Persistent microbial biofilms and filling residuals in "hard-to-reach" areas of the complex root canal system, particularly in the apical region, are more likely to develop post-treatment AP, even in well-treated teeth [36]. Despite the recent advances in endodontic materials and technologies, there is still a need to promote new solutions, overcoming the flaws of the standard disinfecting protocol while fighting the increasing emergence of pathogenic bacterial and fungi resistance. An ideal solvent should also be able to have antimicrobial efficacy, accounting for additional disinfection over the adhered biofilms, exposed by filling materials' dissolution [29]. Thus, an intro investigation was developed in order to evaluate the antimicrobial and antibiofilm activity of MEK/OOil and MEK/TCE, two promising solvent proposals for filling residuals dissolution, on *C. albicans* SC5314 planktonic cells and biofilm. The conventional irrigation of NaOCl and EDTA was used as a reference of the standard recommended clinical procedure.

A preliminary disk diffusion assay commonly used to screen the in vitro antimicrobial activity of new agents was performed. Apparently, all the tested solvents and mixtures exhibited a prelude antifungal effect. Despite the well-known limitations of disc diffusion assay, it easily allowed to compare the solvents tested (Table 1), highlighting the great diffusion ability of OOil and its superior antifungal activity against *C. albicans*. The results are in agreement with other reports, confirming the antimicrobial effect of OOil and TCE, tested in different microorganisms [29,37]. A relevant antifungal activity of both solvent mixtures was also demonstrated, particularly when OOil was present.

The investigation revealed that a 24 h *C. albicans* biofilm exposed to the sequence NaOCl and EDTA still presented some yeast viable cells, which were largely reduced after the exposure to either solvent mixture, MEK/TCE, or MEK/OOil (Figure 1). Despite only including one strain (*C. albicans* SC5314), the results corroborate other investigations with several laboratory and oral clinical isolates [5]. MEK/OOil had a particularly excellent antifungal and antibiofilm activity since it was totally effective, overcoming the "flaw" of the conventional NaOCl and EDTA sequence, emphasized by Alshanta et al. [5]. The quantification of biofilm viable cell counts was further reinforced by the SEM results (Figure 1). The superior performance of solvent mixtures presenting high filling dissolution and low cytotoxicity, previously reported by our team [31], was still reinforced by their antimicrobial activity over planktonic and *C. albicans* biofilm. Our hypothesis is that the synergy obtained by solvent association, improving filling material dissolution [31] could also be applied here, resulting in a superior anti-biofilm effect of MEK/TCE regarding MEK, despite the apparently null antibiofilm activity of the isolated TCE.

To our knowledge, this is the first study with evidence of antifungal and antibiofilm activity of endodontic solvent associations, with a different insight into retreatment procedures. Some limitations should be stressed, apart from those inherent to in vitro studies, such as the influence of the biofilm age or structure [5,12,28] on the efficacy of different endodontic irrigating solutions. The higher antibiofilm effect of both mixtures, particularly MEK/OOil, was clear compared with the standard protocol. The additional retreatment step, incorporating novel endodontic solvents with the dual role of dissolving and disinfecting, might be relevant over persistent biofilms, such as *Candida albicans'*, even whenever filling removal is incomplete. Concerns about the deleterious effect of irrigating/solvent solutions on dentin's mechanical properties [13,38,39], risking tooth integrity or enabling easier bacterial colonization [40], have been emphasized.

Further research should explore other oral microorganisms and multispecies biofilms in an attempt to better simulate the clinical situation. Investigations should also be pursued in order to improve root canal cleanliness in the deepest areas, such as recesses, ramifications, and isthmus.

5. Conclusions

The results of the present investigation constitute the first evidence of the effectiveness of two novel endodontic solvent mixtures, MEK/TCE and MEK/OOil, against biofilms of *C. albicans*. With the goal of developing solvent proposals targeting the most used filling materials, it seems that an additional benefit of disrupting refractory biofilms can also be achieved.

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