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ORIGINAL ARTICLE

Antimicrobial effectiveness of etidronate powder (Dual Rinse® HEDP) and two EDTA preparations against *Enterococcus faecalis*: a preliminary laboratory study

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Abstract

This study aimed to evaluate the antimicrobial and antibiofilm activity of two chelating agents: ethylenediaminetetraacetic acid (EDTA) combined or not with detergents, and etidronic acid combined with sterile saline. The bacterial inhibitory and bactericidal concentrations (MIC and MBC, respectively) were determined on *Enterococcus faecalis* ATCC 4083 strain. Antimicrobial tests were performed on a biofilm model after treatment with the chelating agents at different times (1, 3, and 5 min) using a biofilm eradication concentration (MBEC) and confocal laser scanning microscope (CLSM) assays. Quantification of cell biomass and percentage of live and dead cells in the biomass was assessed for each group. The normality of the distributions for each variable was assessed using the D'Agostino and Pearson's omnibus normality test. The comparison of bacterial viability among groups and between any two groups was performed using the non-parametric Kruskal–Wallis one-way analysis of variance and the Dunn's test, respectively. No significant between-group difference was observed regarding biomass reduction. On the other hand, EDTA combined with detergents displayed a substantial increase of the dead bacteria ranging between 35 and 43%; whereas, the number of cells killed in the control group and in the other treated groups always ranged between 1 and 6%, at all experimental times. The addition of detergents to EDTA can improve its anti-biofilm activity by reducing EPS production and enhancing the killing of sessile bacterial cells. *Clinical relevance* EDTA presents a relevant antimicrobial activity when combined with surface-active agents.

Keywords Biofilm · Chelating agents · Confocal laser scanning microscopy · Detergents · *Enterococcus faecalis*

Introduction

The primary purpose of root canal treatment is to reduce the bacterial load in the root canal system to subcritical levels that are compatible with periapical health [1]. This goal often represents a challenge due to the anatomical complexity of the root canal space [2]. Studies investigating

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the effect of mechanical instrumentation (regardless of the instrumentation technique used) on remaining bacteria and debridement of the root canal have reported poor debridement [3] and inadequate disinfection of the root canal system [4]. Therefore, mechanical instrumentation (with manual or rotary instruments) must be used in conjunction with irrigant solutions.

Sodium hypochlorite (NaOCl) followed by ethylenediaminetetraacetic acid (EDTA), as a final rinse is the irrigation protocol most commonly used by clinicians during endodontic treatment [5] because it may effectively dissolve necrotic tissue, remove the smear layer, and kill microorganisms in the root canal space [6]. It is known that NaOCl alone cannot remove the mineral content of the smear layer that forms on canal walls during root canal instrumentation [7]. Therefore, the use of chelating agents, such as EDTA [8], has been suggested. However, EDTA has some drawbacks: it has a high surface tension [9], which could limit its penetration into dentinal tubules as well as into irregularities of the root canal system such as fins and isthmuses. Furthermore, it retains little or no antibacterial activity [10]. Adding surface-active agents could be a possible way of improving the bactericidal efficacy of EDTA. It has been shown that adding a cationic surfactant as cetrimide to the EDTA (EDTAC) decreases its surface tension [11], increasing its penetrability into the areas of the root canal system inaccessible to mechanical instrumentation, its antibacterial activity and smear layer removal on dentine surface [12].

During root canal irrigation procedure, the combined use of NaOCl and EDTA creates a chemical interaction and an exothermic reaction [13], thus reducing available chlorine in NaOCl solutions up to 80%, and compromising the microbicidal and pulp-dissolving properties of the oxidant solution.

Because of these limitations, recently, a single combined irrigant has been developed to dissolve the organic tissue and remove the smear layer from the root canal system [14].

In 2018, etidronic acid (HEDP), a relatively weak chelator, has been approved for clinical usage (Dual Rinse® HEDP, Medcem Weinfelden, Switzerland). An irrigating solution obtained by dissolving 0.9 g of HEDP powder in 10 ml of NaOCl was found to maintain the free available chlorine for at least 1 h, thereby showing good stability [14]. Some investigations found that antimicrobial and tissue dissolving properties of this combined solution are not impaired with respect to NaOCl alone [15, 16].

Consequently, it has been proposed that this solution could be used as a single irrigant during and after instrumentation, replacing the final rinse with a chelating agent [17].

To obtain a mildly decalcifying solution without any proteolytic and antibacterial properties (without addition to NaOCl), the manufacturer has recommended mixing Dual Rinse® HEDP with sterile physiological saline (1 capsule in 10 mL) for the final rinse of the root canals (https://www.medcem.ch/en/shop/dual-rinse-hedp).

Enterococcus faecalis (*E. faecalis*) has been frequently isolated in persistent root canal infections after failed endodontic treatment compared to primary chronic periapical periodontitis [18]. The main causes of these persistent infections have been attributed to the virulence factors of this bacterium that can maintain periradicular disease as monoinfectant [19], as well as to its ability to forming biofilm in the root canal system. The latter may increase its resistance to mechanical–chemical treatments of the root canal [20], and to antibiotics, medicaments, and some chemical agents [21].

To date, there are only a few investigations concerning the antimicrobial efficacy of EDTA combined with detergents [21, 22]. Instead, no reports are available in the literature about the antimicrobial activity of Dual Rinse® HEDP added to sterile saline solution. However, if microbiological assessments of the clinically approved etidronate powder (Dual Rinse® HEDP) mixed to NaOCl solution have been reported by various studies [17, 23], bacteriological investigations on this chemical agent alone are lacking.

Thus, this laboratory study aimed to compare the antimicrobial activity of Dual Rinse® HEDP + sterile physiological saline with two 17% EDTA solutions: one established without detergents and one with surfactants added.

Materials and methods

Solutions

For this study, three irrigating solutions were evaluated:

1. 17% EDTA (Ogna Laboratori Farmaceutici, Muggiò, Italy);
2. 17% EDTA (Ogna Laboratori Farmaceutici, Muggiò, Italy) plus surface-active agents cetrimide (CTR) and polypropylene glycol (PPG) (Sigma-Aldrich, Milano, Italy), adding each detergent individually or both in combination, as more fully described below.
3. 0.9 g Dual Rinse® HEDP (Medcem, Weinfelden, Switzerland) + sterile physiological saline.

The above EDTA solutions were used because they have been certified by TÜV Service CE 0123 (TÜV SÜD Produkt Service GmbH, Ridlerstrasse 65, 80,339 Munich, Germany), and recommended for clinical use according to ISO 10993-5:2009 and ISO 10993-10:2010 (Test Report n. 18/000458102) by Mérieux NutriSciences Chelab Srl (Resana, Italy). All 17% EDTA solutions were freshly prepared by the manufacturer, and the pH of the solutions was adjusted to 7.5. The mixed solution Dual Rinse® HEDP + sterile physiological saline was obtained by adding

0.9 g of Dual Rinse® HEDP powder (mean content per capsule) to 10 mL of sterile physiological saline (0.9% NaCl solution) obtaining a 9% solution with a pH equal to 11.4. The pH of the three solutions was measured using a calibrated microelectrode (827 pH lab, Metrohm Italiana Srl, Origgio Varese, Italy).

Bacterial strains and culture media

Enterococcus faecalis (*E. faecalis*) strain ATCC 4083 was obtained by American Type Culture Collection (ATCC) in frozen stock and stored at − 80 °C before analysis. Then, before use, the strain was thawed and reconstituted in Tryptic Soy Agar (TSA, Biomérieux, Marci l'Etoile, France) for 24 h at 37 °C.

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antimicrobial activity of chelating agents under investigation was assessed by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values against the microbial strain described above. The MIC was established by the broth microdilution method, following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/MIC_testing/Edis5.1_broth_dilution.pdf).

Briefly, a microbial suspension at an optical density equal to 0.5 McFarland standard in Muller Hinton broth (MHB, Biolife Italiana S.r.l. Milano, Italy) was prepared from *E. faecalis* ATCC 4083 strain. After obtaining a microbial load of 5×10^6 CFU/ml using appropriate dilutions, 10 µl of each suspension was inoculated in a 96-well microplate containing 90 µl of a serial twofold dilution of the chelating solutions (17% EDTA, 17% EDTA + CTR + PPG, 17% EDTA + CTR, 17% EDTA + PPG, Dual Rinse® HEDP + saline). Bacterial growth in the control group was performed by inoculating the *E. faecalis* suspension in MHB. MIC values were read after 24 h of incubation at 37 °C. The minimum inhibitory concentration, defined as the lowest concentration of an antimicrobial substance able to kill 99.9% of the initial inoculum, was determined by subculturing 10 µl of microbial suspension from wells showing no visible growth in the MIC tests. Similarly, MBC values were read after 24 h of incubation at 37 °C [24].

Minimum biofilm eradication concentrations (MBEC)

MBEC assay was employed to assess the lowest concentration of a medicament that prevents visible growth in the recovery medium used to collect biofilm cells. MBEC evaluation of the chelating agents was carried out using the MBEC™ High-throughput (HTP) assay (Innovotech, Edmonton, Alberta, Canada) and adapted for *E. faecalis* as previously reported [10]. The wells of an MBEC device were filled with 150 µL of Brain Heart Infusion (BHI) broth (Biolife Italiana S.r.l. Milano, Italy) inoculated with 10^7 CFU/mL of *E. faecalis* resuspended from an overnight culture on Tryptic Soy Agar (TSA Biolife Italiana S.r.l. Milano, Italy), and then incubated for 24 h at 37 °C to establish a biofilm on the pegs located on the lid of the device. After the incubation, the lid was placed for approximately 30 s in a sterile 96-well microplate containing 200 µL of sterile saline solution (rinse plate) to remove unattached cells. Afterward, the lid was placed on a "challenge plate" containing 200 µL of a serial twofold dilution of the five chelating solutions for 1′, 3′ or 5′ while agitating on an orbital shaker, and moved again in a new rinse plate for 30′ to neutralize the test solutions.

Consequently, the lid was placed in a new 96-well microplate containing 200 µL of BHI broth and placed in a dry stainless steel tray which sits in a water bath and sonicated at maximum power for 10′ to dislodge the remaining biofilm on the pegs. The lid was then removed, replaced with a non-pegged lid and the plate incubated overnight at 37 °C. MBEC values were determined by visually checking the wells for turbidity. Clear wells indicated a full biofilm removal.

E. faecalis biofilm treatment—confocal laser scanning microscopy (CLSM) analysis

The amount of biofilm was quantified using CLSM analysis. *E. faecalis* biofilm was cultured on uncoated 10-mm-diameter glass slides (VWR International Srl, Milano, IT) placed in 24-well microplates for 48 h, by inoculating bacterial cells in 1 ml of BHI broth to a final concentration of 10^7 CFU/ml. At the end of the incubation time, the culture medium was removed, and two washes with sterile saline were performed to remove non-adherent bacteria. Afterward, the biofilm was treated with each of the testing solutions for 1, 3, and 5 min (controls were treated with sterile saline). Then, the solutions were removed, and the wells were washed twice with saline. Because the addition of PPG did not change the MIC, MBC and MBEC values of the EDTA solution, while CTR has shown the antibacterial action of this combination, the two previous associations (EDTA + PPG and EDTA + CTR) have not been evaluated in the CLSM assay. Thus, only three chelating agents alone or in combination with detergents (17% EDTA, 17% EDTA + both detergents CTR and PPG, Dual Rinse® HEDP + saline) in the CLSM analysis were considered.

Glass slides were then stained with Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Thermo Fisher Diagnostics

SpA, Rodano, IT), prepared according to manufacturer instructions. Each sample was stained with 30 µL of staining solution by incubating for 15 min at room temperature in the dark.

Subsequently, stained biofilms were thoroughly rinsed in physiological solution and the images acquired with a Confocal Laser Scanning Microscope TCS SP8 (Leica Microsystems CMS GmbH, Mannheim, DE) using a 20 × dry objective (HC PL FLUOTAR 20 × /0.50 DRY). Sequential optical sections were gathered along the *z* axis for the entire thickness of the biofilm. Images from a minimum of three random areas were acquired for each of three replicates by applying the same laser intensities. The selected images were processed with Las X (Leica Microsystems CMS GmbH, Mannheim, DE) and analyzed with Fiji software (Fiji, ImageJ, Wayne Rasband, NIH) after applying a standardized background threshold. The quantification of cells biomass was expressed in μm^3. The percentage of live and dead microorganisms in the biomass was assessed in each group.

A preliminary study, not doing part of the present investigation, was carried out in a separate set of experiments on ten teeth to verify the smear removal ability of the three chelators used in the CLSM analysis (data not shown but available as Supplementary Electronic Material). Nine intact single-rooted human teeth, after the shaping and cleaning of the root canals, were divided into three groups (3 samples each) depending on the final rinse used. A supplemental tooth served as a positive control (final rinse with distilled water). Subsequently, the specimens were divided into two halves, that were coated with gold and examined using scanning electron microscopy (SEM) Nova NanoSEM 450, (FEI Company, Eindhoven, The Netherlands) at 2000 × magnification.

Statistical analysis

GraphPad Prism 5.0 (GraphPad San Diego, CA, USA) was utilized as the analytical software. The normality of the distributions for each variable was assessed using the D'Agostino and Pearson's omnibus normality test. Because the data did not show a normal distribution, the non-parametric Kruskal–Wallis one-way analysis of variance and the Dunn's test were used to assess the differences among groups and between any two groups, respectively. Values of $P < 0.05$ were regarded as significant.

Results

Antimicrobial activity by broth microdilution.

In Table 1, MIC and MBC values of the tested solutions are shown. Dual Rinse® solution was able to inhibit *E. faecalis* growth only when not diluted, but still, such concentration was not able to kill all the cells of the inoculum. EDTA did inhibit bacterial growth from the concentration of 42.5 g/l (1:2 dilution) and killed all the cells from 85 g/l (1:1 dilution). The addition of PPG to EDTA increased neither the inhibitory nor the bactericidal activity. When cetrimide was added to the EDTA solution, growth was inhibited from a dilution of 1: 2048, and no residual live cells were found from a dilution of 1: 256. Again, the addition of PPG did not change MIC and MBC values of the EDTA solution combined with detergents.

Minimum biofilm eradication concentrations (MBEC)

While EDTA alone, EDTA with PPG and Dual Rinse® irrigant were not able to dislodge the biofilms formed on the pegs even at the higher concentrations, the addition of cetrimide could entirely remove the attached sessile cells when diluted 1:64 if treated for 1′, and diluted 1:128 when treated for 3′ and 5′ (Table 1).

Treatment of pre-formed *E. faecalis* biofilm

When the effect of the tested solutions on pre-formed enterococcal biofilm was investigated using the CLSM assay, no significant differences were observed on biofilm biomass reduction, at all the times of exposure (Figs. 1, 2a). The biomass reduction ranged from 14 to 35% in the different treatment groups. On the other hand, a statistically significant increase in dead/live bacteria ratio was observed for EDTA + detergents group as compared to the control group

Table 1 Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and Minimum biofilm eradication concentration (MBEC) of the tested solutions

	Dilutions				
	MIC	MBC	MBEC 1'	MBEC 3'	MBEC 5'
17% EDTA + CTR + PPG	1:2048	1:256	1:64	1:128	1:128
17% EDTA	1:2	1:1	NA	NA	NA
17% EDTA + CTR	1:2048	1:512	1:64	1:128	1:128
17% EDTA + PPG	1:2	1:1	NA	NA	NA
Dual Rinse + saline	undiluted	NA	NA	NA	NA

CTR cetrimide, *PPG* polypropylene glycol, *NA* no activity

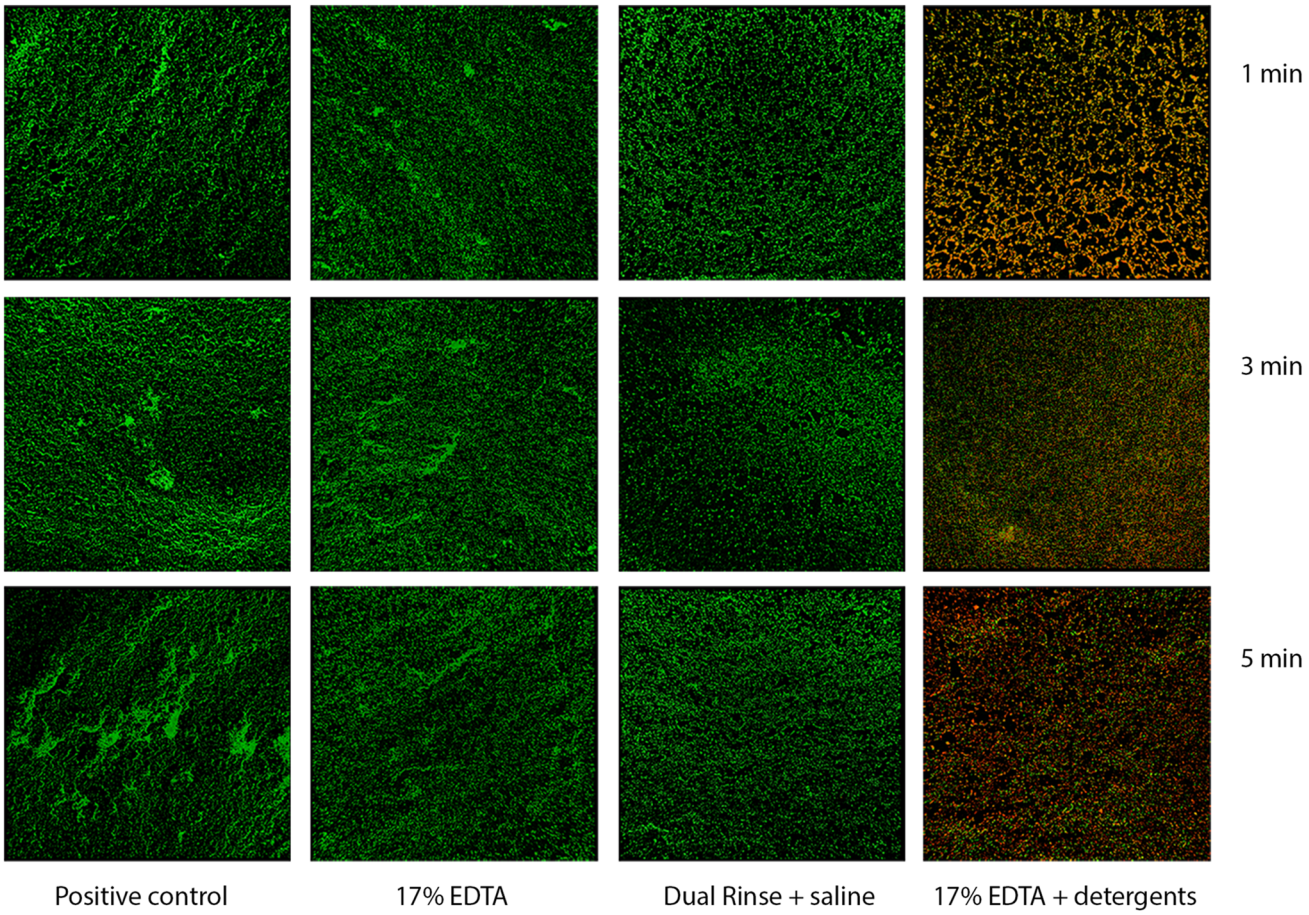

Fig. 1 CLSM 3D images of biofilms treated with three solutions and positive control (saline solution) at different times of exposure. Live cells are seen in green, and dead cells are seen in red. Each picture represents an area of 351 × 351 µm

and the groups treated with only EDTA and Dual Rinse® irrigant (Fig. 2b). Indeed, the percentage of dead cells in the biofilms treated with EDTA + detergents solution increased from 35 to 43% with the increment of time of exposure; whereas, the number of cells killed in control group and the other treated groups always ranged between 1 and 6% (Fig. 2b).

Our preliminary SEM study showed the dentinal walls covered with the smear layer in positive control and after final irrigation with HEDP, without open dentinal tubules exposed. On the contrary, the final rinse with 17% EDTA and 17% EDTA with detergents did not show the smear layers in the coronal and middle third of root canals. The smear layer, instead, covered the dentinal wall in the apical third of all the samples, regardless of the irrigating solution used (supplementary material).

Discussion

In the current study, the antimicrobial activity of some chelating agents was evaluated on *E. faecalis* strain ATCC 4083. The reason for choosing this strain, instead of ATCC 29212, the most used as test microorganism, was due to the fact that it was primarily isolated from the root canal of pulpless tooth [25], while the source of strain ATCC 29212 was the urinary tract (https://www.atcc.org/products/all/29212.aspx), thereby simulating the clinical condition as close as possible.

Limited to our knowledge, very few studies have evaluated the antibacterial properties of 17% EDTA with detergents added, as highlighted above, and none of Dual Rinse® HEDP powder dissolved in sterile saline solution so far. Dunavant et al. [21] demonstrated that Smear Clear (Kerr Endodontics, Orange, CA, USA), a mixture of EDTA, cetrimide and Triton X-100, significantly reduced *E. faecalis*

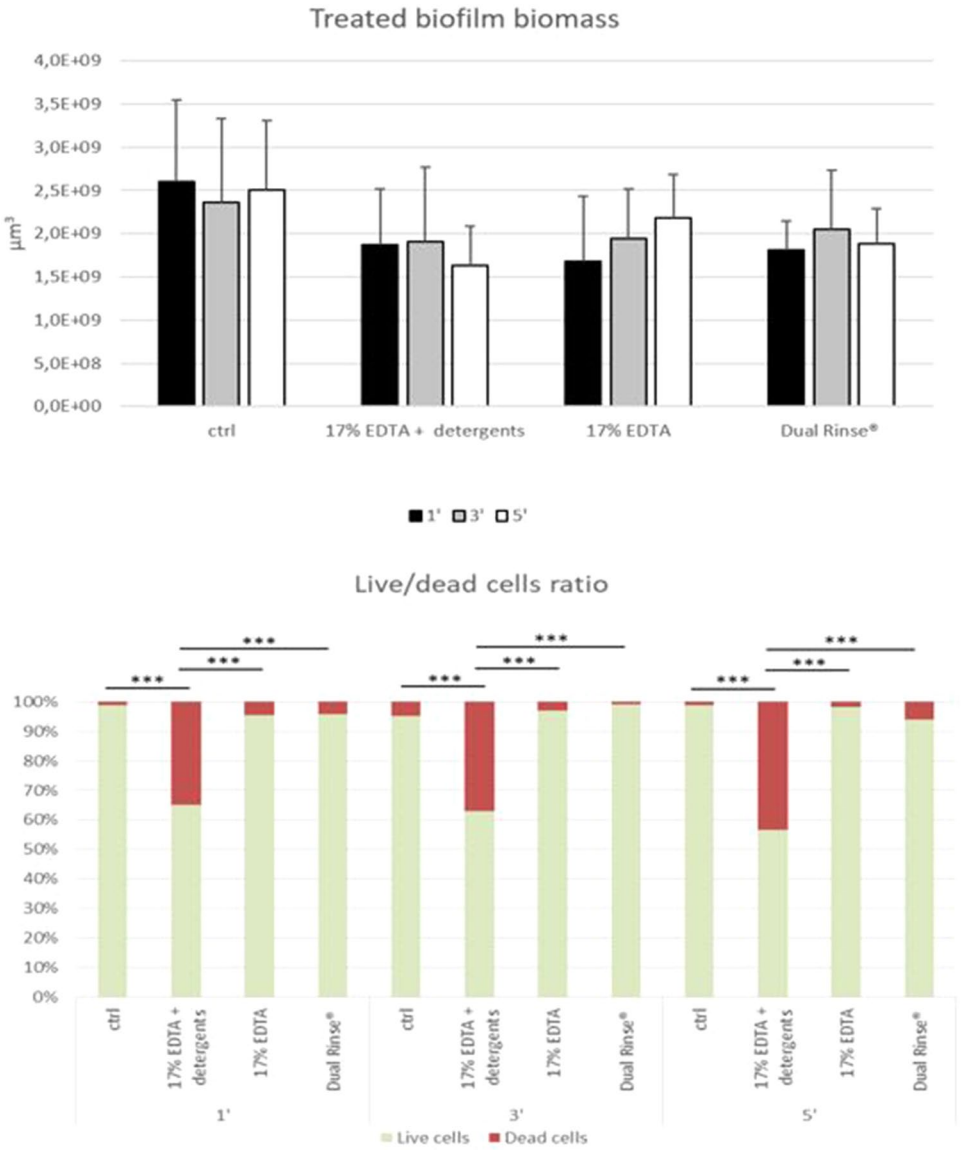

Fig. 2 CLSM analysis of *E. faecalis* biofilm upon exposure to the tested solutions. **a** *E. faecalis* biofilm biomass expressed in μm^3. Black bars = 1′ treatment; gray bars = 3′ treatment; with bars = 5′ treatment. **b** Live/dead cells ratio in the treated biofilms. Error bars refer to dead cells standard deviation. Green = live biofilm cells; red = dead biofilm cells. ***$P < 0.001$

compared to 17% EDTA. The antimicrobial action mentioned above is probably due to the addition of the cationic surfactant cetrimide, which has bactericidal and fungicidal properties [26].

Lately, this better antibacterial action by adding the cetrimide to other chelating agents has been reported [22, 27], confirming those results.

HEDP exists as disodium (Na_2) and tetrasodium (Na_4) salts, which can be dissolved in water to form aqueous irrigants of different pH [15]. Some researchers have shown that HEDP solutions prepared from the tetrasodium salts were alkaline (pH 11.3) decreasing its decalcifying effect, while the counterparts made from the disodium salts were acidic (pH 4.6) increasing its effectiveness in removing smear layer [28]. The same authors have also highlighted that smear layer removal is influenced by the amount of sodium contained in the salts to prepare the respective solutions and thus pH. Alkaline solutions containing these chemicals remove less calcium and smear layer from root dentine than more neutral or acidic counterparts.

Similar to HEDP tetrasodium (Na_4) salts, EDTA tetrasodium salt ($EDTANa_4$) at alkaline pH is weaker in the removal of the smear layer than disodium EDTA. The better smear layer removal efficacy of EDTA solutions may be attributed to their lower or neutral pH [29]. A high pH negatively impacts the EDTA effectiveness due to excess hydroxyl ions, which significantly reduce the dissociation of smear layer hydroxyapatite, thus limiting the number of free calcium ions that EDTA can chelate. The high pH significantly inhibits and reduces the dissociation of hydroxyapatite and likewise, the demineralization efficacy of EDTA [29]. Moreover, the results of the above study highlighted

that EDTA solutions at an alkaline pH were less effective in removing the smear layer than solutions at a neutral pH. This is also because, at low or neutral pH, the binding of Ca^{2+} tends to increase the dissociation of hydroxyapatite and its availability for chelating.

Previous researches showed somewhat conflicting results on the biofilm disruption or antimicrobial effect of chelators on the biofilm ranging from none to some effect [10, 30]. This could be related to the short application time used in those studies. This limited application time was recommended because the exposure to these agents for more than 5 min may cause peritubular and intertubular dentin erosion [31].

According to Wang et al. [32], in infected root canals, the smear layer should be removed to maximize the effect of disinfecting solutions against bacteria in the dentinal tubules. In that study, it has been shown that the bacterial killing by irrigants tested was significantly lower when the smear layer was present than when it was not. For this reason, EDTA solutions at neutral pH were herein used. EDTA itself is not antimicrobial, as herein showed, when exposed to *E. faecalis* cells in planktonic killing tests (MIC, MBC) [32] but can exert antimicrobial activity by chelating divalent cations (Ca^{2+}, Mg^{2+}) necessary for growth and membrane stability and may also display anti-biofilm activity by reducing EPS production and/or enhancing the detachment of bacterial cells from the biofilm [33].

Divalent metals are involved in all aspects of microbial growth, metabolism differentiation, and survival [34], then their chelation by EDTA can result in weakening of the exopolymeric matrix structure of biofilms, facilitating its removal.

In line with the previous investigation [33], a recent study highlighted that EDTA could exert an antimicrobial activity by detaching the most portion of bacteria from *E. faecalis* biofilm [35].

Previously, it was highlighted that EDTA per se is mainly known for its biofilm-dispersing properties with no or little antimicrobial effect. The only antibacterial property recognized to EDTA is to weaken the bacterial cell membrane without killing the cell and promote the biofilm detachment [35].

These data have been confirmed herein. Interestingly, CTR added to EDTA revealed the most prominent effect, being inhibitory even when diluted 1:2048 and bactericidal at 1:512. Likewise, EDTA combined with CTR was observed once again as the most active agent, being able to completely remove *E. faecalis* biofilm from the MBEC pegs diluted 1: 64 and 1:128, respectively, when applied for 1′, 3′ and 5′ (Table 1). Then, this combination, maintaining its chelating properties without interfering with CTR antimicrobial and cleansing activity, could be useful for clinical use.

Thanks to these properties, the use of the divalent cation chelator EDTA has been approved in humans as a treatment or preventing biofilm formation in some fields of medicine [36, 37].

The reason why Dual Rinse® HEDP was investigated in its original formula, without the addition of surfactants, was to follow the manufacturer's recommendations, as mentioned previously (https://www.medcem.ch/en/shop/dual-rinse-hedp). The antimicrobial activity of established EDTA solutions containing CTR has been studied earlier by cultural method on *E. faecalis* biofilms, calculating the percentage kill of viable bacteria following treatment with these agents [21, 22].

To date, a method allowing a complete analysis of biofilm does not exist: quantification of EPS, viable bacteria, total live cells number, thickness and roughness, elasticity of a specific biofilm require more than a single method. Due to this limitation, herein, to quantify the live/dead cells and antibacterial activity of the biofilm exposed to the solutions, MBEC and CLSM methodologies were used [38]. Presently, instead, two different methodologies of analysis were utilized to verify the antimicrobial activity of the chelator solutions before and after exposure: the microbiological cultures and CLSM analysis on planktonic and biofilm forms (Table 1, Fig. 1). The results of the present study have shown that EDTA + detergents combination was able to reduce the total number of viable cells and was associated with the highest number of dead cells when compared to both groups and positive control (Fig. 2a, b).

Interestingly, though in the present study no significant differences were observed on biomass reduction (Fig. 2a), a statistically significant increase ($P < 0.001$) in dead/live bacteria ratio was seen in the EDTA + detergents treated group compared to the control group and the groups treated with only EDTA and Dual Rinse® irrigants (Fig. 2b). This was probably due to the addition of the detergents.

When surfactants are added to irrigants, they reduce the surface tension [39] and enhance the antibacterial effect of the solutions by a residual antibacterial activity, increasing the resistance to microbial regrowth in the root canal system [40]. Some studies stated that cetrimide, a cationic surfactant, in addition to a proven bactericidal activity and thanks to the capacity to reduce the surface tension of irrigants [41], can decrease the mechanical stability of the biofilm by destabilization of the cohesive forces of the biofilm. This aspect probably depends on the interaction between the chemical structure of the molecule and the anionic properties of the extracellular polymeric substance (EPS) surrounding the biofilm [42]. All these features can contribute to greater efficiency of irrigating solutions where detergents are added, as highlighted in the current investigation, and explain the best results obtained compared to both surfactant-free solutions. The addition of non-ionic detergent polypropylene

glycol (PPG) to EDTA was not contributive. It increased neither the inhibitory activity nor the bactericidal activity (Table 1), only further reduced the surface tension of the experimental solution compared to the addition of cetrimide (data unpublished).

It has been shown that a saline HEDP solution did not present any significant effect against *E. faecalis* biofilms after 10 min of exposure, contrary to its high antibacterial action inside dentinal tubules at the same exposure time [17]. Then, these results could be attributed to the different density of cells found in dentinal tubules and biofilm biomass [17]. As herein confirmed, HEDP solution presents an alkaline pH (Table 1). The higher biomass probably presents increased resistance to the alkaline stress and the ability to neutralize the alkaline pH in comparison with bacteria inside dentinal tubules [17]. Moreover, *E. faecalis* is known to retain an exceptional recalcitrance to elevated pH, also when in planktonic state [43], and the genus *Enterococcus* has been identified more frequently from clinical samples cultured in buffer-enriched BHI broth adjusted to pH 9.0–11.0, compared to the many alkali-resistant bacterial species isolated in infected root canals dentine [44].

Our laboratory-based study results revealed that the EDTA solution with added detergents could induce a significant increase in antimicrobial action, in agreement with the results of Ferrer-Luque et al. [22]. Moreover, a similar or better smear layer removal of this combined solution was also observed when compared to EDTA and HEDP (supplementary material available). Noteworthy, cetrimide does not influence the demineralization capacity of EDTA compared to other EDTA-based solutions with added surfactants (SmearClear and QMiX) [45], as also noted previously [46]. Combinations of EDTA and surfactants could be useful in clinical practice, thanks even to its low surface tension that allows a more effective debris removal and penetration into the root canal system, as stated in Endodontic literature. Although the primary purpose of a chelator solution, intended for endodontic use, is to remove the smear layer, according to Zehnder [47], an ideal irrigant should also have a broad antimicrobial spectrum and high efficacy against microorganisms organized in biofilms.

Further studies using a multi-species biofilm model would be useful to confirm the promising results observed in the current study. Besides, studies concerning biocompatibility and the removal of accumulated hard-tissue debris (AHTD), produced during mechanical preparation procedures, should be conducted on new chelating solutions for their safe and efficient clinical usage. These aspects will be addressed in the second part of this preliminary study.

Within the limits of the results obtained in the present study, HEDP solution + sterile physiological saline should not be recommended as a single irrigant solution, due to no antibacterial efficacy and no or limited ability to remove the smear layer. Our data confirmed that HEDP has not antimicrobial efficacy; it was reported to function as stabilizer or sequestrant in the mixtures of the components, immobilizing metal ions [48].

Therefore, due to its established properties, its use should be recommended only in association with NaOCl without interfering its antimicrobial properties [17], or alone in regenerative endodontic cases as suggested in previous study [49].

Conclusions

Under the conditions of the current study, EDTA with surface-active agents displayed some peculiar features among the tested endodontic irrigants. The 17% EDTA solution with detergents increases the number of dead cells in the biofilm. Its increased bactericidal activity depends solely on the addition of cetrimide, being polypropylene glycol not contributive to this purpose. HEDP + saline alone shows no or little antibacterial activity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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