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Original Research Article

Effect of the association of different substances at the physical and antimicrobial properties of the EDTA

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Abstract

Introduction: We aimed to assess the physical, chelating, antibacterial properties and cytotoxicity of EDTA used with and without antibacterial additives. **Material and methods:** Solutions of 17% EDTA with or without additives together with 2.5% sodium hypochlorite and saline control were tested. The antibacterial properties of the solutions were assessed using a biofilm which was inoculated in human dentine either before exposure to irrigating solution or after. The smear layer removal capacity was tested by using scanning electron microscopy (SEM). The irrigants were also evaluated for dentine wettability by the sessile drop method, and the surface tension of solutions was verified by the pendent drop test. The effect of irrigating solutions on fibroblast cell viability was assessed by reducing MTT assay. **Results:** The addition of antibacterial

substances to EDTA resulted in improving its antibacterial action against *Enterococcus faecalis* biofilm, without compromising the EDTA chelating action or elevating its cytotoxicity. Lower surface tension and better wettability of the irrigants was obtained by sodium hypochlorite or by adding benzalkonium chloride or N-acetylcysteine to EDTA. **Conclusion:** EDTA-benzalkonium chloride showed effectiveness not only preventing the formation of biofilm and root canal re-infection, but also against *Enterococcus faecalis* biofilm itself. It also lowered EDTA surface tension and allowed better dentine wettability, being outstanding compared with the other chelator solutions. Therefore, the use of this association as a final rinse in endodontic treatment should be further studied.

Introduction

The irrigation protocol is an important step which effects the success of root canal therapy [11, 36]. One of it goals is the removal of smear layer represented by debris on the dentin wall arising from organic and inorganic matter formed during root canal instrumentation.The smear layer promotes dentin tubule obliteration, making it difficult for the intracanal medication and sealers to penetrate [13], and may increase the adhesion of microorganisms initiating the process of biofilm formation [34]. Used throughout the biomechanical preparation, sodium hypochlorite is recommended as the main irrigant due to it broad antimicrobial spectrum and capacity for dissolving organic matter, such as pulp remenants, bacterial components and their by-products [36]. The chelating agent disodium or trisodium ethylenediaminetetracetic acid (EDTA) is used as adjunct irrigant to remove the inorganic portion of the smear layer.[20]. The low antibacterial ability of EDTA, when compared with sodium hypochlorite or chlorhexidine necessitates a third stage of final irrigation with these solutions, with the purpose of increasing disinfection of root canal system [10, 33, 35].

Although sodium hypochlorite is the gold standard for root canal irrigation, it is a dangerous chemical if ingested and causes severe bruising when it comes in contact with the soft tissues mainly at high concentrations [4]. Furthermore, sodium hypochlorite causes degradation of the dentin organic matrix [28]. Improvement in the antibacterial properties of EDTA would result in a reduction in use of the sodium hypochlorite and the use of one final irrigating solution achieving both the chelating and antimicrobial effect. The addition of antibacterial agents such as benzalkonium chloride, chlorhexidine and N-acetylcysteine would potentially achieve this [14, 21, 22]. The low surface tension of these mixtures, could increase EDTA wettability, leading it to reach deeper areas into the dentinal tubules [8] that could contain bacteria. The removal of smear layer in these areas could also improve the adhesion of filling materials to the dentin.

The aim of this study was to investigate the antibacterial action, smear layer removal, wettability, surface tension and cytotoxicity of EDTA and modified EDTA chelating agents. Furthermore, the wettability of dentin and adhesion of bacteria were analyzed after treatment with the irrigant solutions. The null hypothesis was that the modified EDTA solutions would show similarity to one another and to pure EDTA.

Material and methods

Five irrigating solutions were investigated. These included G1 – 17% EDTA (Biodinâmica Química e Farmacêutica LTDA, PR, Brazil); G2 – 17% EDTA + 1% benzalkonium chloride (Bauru Fórmulas, Bauru, SP, Brazil); G3 – 17% EDTA + 1% N-acetylcysteine (Bauru Fórmulas, São Paulo, Brazil); G4 – 17% EDTA + 2% chlorhexidine (Bauru Fórmulas, Bauru, SP, Brazil); G5 – 2.5% sodium hyphoclorite (NaOCl) (Rioquímica, São José do Rio Preto, SP, Brazil) and G6 – saline solution.

Antibacterial testing

The microbiological procedures were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil).

Specimen preparation

One hundred and twenty dentine blocks were obtained from the cervical area of bovine central

incisors with fully developed roots, by using trephine drills in 4.0-mm diameter (Neodent, Curitiba, PR, Brazil), under copious irrigation. The incisors were positioned laterally to provide access to the most flattened portion of the root. The root was perforated with a trephine attached to a contra-angle handpiece positioned perpendicular to the teeth passing through the mesial and distal dentine walls, thus obtaining two dentine blocks measuring 4 mm x 1.2 mm (diameter x thickness). The dentine surface of the discs were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 500 and 800 grit SiC papers (Buehler, Lake Bluff, IL, USA) on the pulp surface to smoothen it. The smear layer formed during dentine specimen preparation was removed by submerging the specimens in 17% EDTA for 5 min for the biofilm assay. After this the specimens were sterilized by autoclaving at 121° for 20 minutes. For the adhesion test the specimens were autoclaved after being polished, with no treatment.

Antibacterial test for E. faecalis *biofilm*

For *Enterococcus faecalis (E. faecalis)* biofilm, 15 μL standard strain (ATCC 29212) was put into 3 ml sterile brain heat infusion (BHI) broth (Oxoid, Basingstoke, UK) at 37º, for growth overnight in aerobiosis. After this, bacterial density was adjusted at 10^8cells/ml for *E. faecalis* (ATCC 29212) in a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density (OD) of 1 at 600 nm according to the 0.5 MacFarland standard. A dentine block + 100 μL of *E. faecalis* + 1900 μL of BHI was put into each well of a 24-well multiwell plate (*n = 10*). For biofilm growth, all plates were incubated aerobically at 37ºC for 21 days. The BHI was refreshed every 2 days. After the incubation period, the infected specimens were washed with 1 ml of distilled water to remove loosely adherent planktonic bacteria. Then they were randomly divided into five groups according to the experimental solutions. The contact test was performed by immersing dentine specimens in 5 ml of the experimental solutions for 5 minutes. One group remained without treatment as a control. After the established time of contact with the solutions, the blocks were washed with phosphate-buffered saline (PBS).

Adhesion of E. faecalis *to the dentine surface*

After sterilization the dentine blocks (*n = 10*) were treated with 5 mL of the irrigant solutions for 5 minutes, and then neutralized with saline solution. For specimen contamination, *Enterococcus faecalis* standard strain (ATCC 29212) was grown overnight at 37º in aerobiosis, then the bacterial density was adjusted to 10^7cells/ml (as described in section "Antibacterial test for *E. faecalis* biofilm"). After this, a dentine block + 25 μL of *E. faecalis* + 975 μL of BHI was put into each well of a 24-well multiwell plate for a period of one hour. The specimens were abundantly washed with 2 ml of phosphate buffered saline (PBS) to remove the microorganisms not adhered to the surface of the block.

Microbiological analysis

After washing the dentine blocks (prepared in sections "Antibacterial test for *E. faecalis* biofilm" and "Adhesion of *E. faecalis* to the dentine surface") with phosphate-buffered saline (PBS), they were stained with 15 μL of the SYTO 9/propidium iodide dye (Live/Dead, Baclight, Invitrogen, Eugene, OR) for 15 minutes in a dark environment. Then they were washed again, and directly observed using an inverted confocal laser scanning microscope (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Two confocal "stacks" of random areas were obtained for each specimen using a 40x oil lens. In total, there were 10 specimens per group, therefore, 20 stacks for each group. For quantification purposes, bioImage_L software (www.bioImageL.com) was used to calculate the total biovolume and the percentage of green (live cells) found after the antibacterial treatment.

Smear layer removal

Dentine blocks were obtained from bovine maxillary incisors by using a 4 mm trephine drill (Neodent, Curitiba, PR, Brazil) under copious irrigation, as described in section "Specimen preparation". The dentine surface of the discs were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 300 and 600 grit SiC papers (Buehler, Lake Bluff, IL, USA) for 30 s each to produce a standardized smear layer. To prove the presence of smear layer on dentine walls, low vacuum scanning electron microscopy Aspex Express SEM (FEI Europe, Eidhoven, The Netherlands) images were captured at 550x magnification with a 20 keV beam. Subsequently the blocks were randomly divided into 5 Groups (*n = 10*), treated for 5 minutes with the respective irrigating and chelating agents. As the device used did not require the specimens to be sputter coated, new images were captured (final images) by SEM from the same specimen, according to the previously described parameters.

The presence of smear layer, was scored by three previously calibrated and blinded evaluators assessing the images obtained: Score 1 – no smear layer, all dentinal tubules open; Score 2 – small amount of smear layer, more than half of the dentinal tubules open; Score 3 – homogenous smear layer covering the root canal wall, less than half of the dentinal tubules open; Score 4 – complete root canal wall covered by a homogeneous smear layer, no open dentinal tubules. The difference in the quantity of smear layer before and after use of the irrigant solutions was measured, and corresponded to the value of cleaning the dentine block.

Determining the contact angle measurement by the sessile drop method (Wettability)

Slabs dentine slices measuring 7 × 7 × 1.5 mm (length × width × thickness) were obtained from crowns of bovine incisor teeth. The dentine surface of the discs were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 1200 grit SiC papers (Buehler, Lake Bluff, IL, USA) for 30 s to obtain a flat surface. Then, the specimens were immersed in distilled water and submitted to ultrasonic agitation for 1 min to remove the residues from polishing.

The contact angle measurements were taken with a Goniometer (Drop Shape Analyzer DSA25B) coupled to a Progressive 1/3""CCD camera with filter, high performance acquisition plate, optical length and fixed focus system with a field of view of 11 X 8.2 mm, manual dosage system, syringe, needles and measurement software.

This occurred in two ways: Test 1 – a sessile drop of 1.0 μL of test solution at ambient temperature was dispensed on the dentine specimen surface with the aid of a micropipette; Test 2 – a sessile drop of 1.0 μL of physiological solution at ambient temperature was dispensed from a micropipette into the dentine specimen surface (treated for 5 minutes with the solutions in question) and then dried in an oven. The software measured the angle of contact formed.

In both tests, each dentine specimen ($n = 10$) was analyzed in triplicate. Images were captured 30 s after water deposition using a microvideo system. The images acquired of the static contact angles between the dentine surface and irrigants were determined using the software SCA 20 (DataPhysics Instruments GmbH).

Surface tension determination by the pendant drop method

The contact angle measurements were taken with a Goniometer (Drop Shape Analyzer DSA25B) coupled to a Progressive 1/3""CCD camera with filter, high performance acquisition plate, optical length and fixed focus system with a field of view of 11 X 8.2 mm, manual dosage system, syringe, needles and measurement software. One 1.0 μL drop of the irrigant agents in question was dispensed from the needle. Before the drop fell, the computer program calculated the surface tension or interfacial tension by studying the profile of this pendant drop, in a process that could be divided into three parts: acquiring the image and extracting the contour, smoothing the contour of the drop, and finally calculating the surface or interfacial tension.

Cytotoxicity analysis

Initially, the following stock solutions were prepared by serial dilutions (undiluted, ½, ¼, ⅛) in Dulbecco's modified eagle medium (DMEM) obtaining the following concentrations: Solution 1: EDTA (17%, 8.5%, 4.25%, 2.125%); Solution 2: EDTA (17%, 8,5%, 4,25%, 2,125%) + benzalkonium chloride (1%, 0.5%, 0.25%, 0.125%); Solution 3: EDTA (17%, 8,5%, 4,25%, 2,125%) + N-acetylcysteine (1%, 0.5%, 0.25%, 0.125%); Solution 4: EDTA (17%, 8.5%, 4.25%, 2.125%) + chlorhexidine (2%, 1%, 0.5%, 0.25%) and Solution 5: NaOCl (2.5%, 1.25%, 0.625%, 0.312%).

Cell culture

For the assessment of cell expression, murine NIH/3T3 fibroblasts (ATCC) were grown in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For subculture, they were incubated with trypsin (0.25%) for 3 minutes at 37°C, followed by trypsin inactivation with medium containing 10% FBS. After centrifugation at 1,200 rpm for 5 minutes, the pellet was resuspended in the respective media and the cells were cultured in bottles for further experiments. Cells were incubated at 37°C in a humid atmosphere containing 5% CO_2. For cell viability assay, $1x10^4$ cells / well were plated in 96-well plates. After 24h of incubation, the culture medium was replaced with respective 200 μL of irrigating solutions concentrations for period of 3h. Positive and negative control groups

were also carried out, the cells being treated, respectively, with 10% FBS medium and filtered ultrapure water. Control groups were not exposed to the irrigating solutions.

MTT assay

After treatment time (3h), a solution containing 0.5 mg MTT per ml of medium was added to the plate and they were incubated in a humid atmosphere at 37ºC for 4h. After removal of the MTT, dimethylsulfoxide (DMSO) was added for 30 minutes at room temperature. Absorbance was determined by optical density spectrophotometer with 562nm filter (FluoStar OPTIMA, microplate fluorescence reader) [17].

Statistical analysis

The data of all tests were submitted the Kolmorov-Smirnov test to evaluate the normality. Only the wettability test values showed normal distribution. For wettability, the Anova and Tukey tests were used to compare the groups. In the other tests, the groups were compared using the Kruskal-Wlallis and Dunn tests. The level of significance adopted was 5%. The statistical package used was GraphPad prism 6.0.

Results

The percentage of viable cells and biovolume of *E. faecalis* biofilm after 5 minutes treatment with the EDTA-based solutions are presented in table I and represented in figure 1 by confocal laser scanning microscopy images. The EDTA without additives and saline solution had the lowest antibacterial action against *E. faecalis* biofilm ($P < 0.05$). Both groups had no difference with EDTA + chlorhexidine. Sodium hypochlorite was the most effective in killing bacteria in biofilm and in diminishing the biovolume values of biofilm. There were no differences for the latter and EDTA + benzalkonium chloride or EDTA + N-acetylcysteine No biofilm dissolution in specimens irrigated with chelators was present ($P>0.05$).

Table I also shows the median, minimum and maximum values of bacteria adhesion and viability after 1 hour of contamination on irrigant coated dentine blocks. The bacteria adhesion was avaliated by biovolume of bacteria after extensively irrigation with PBS to detache the non-adherent cells using confocal laser scaning microscopy and live and dead dye, represented by figure 2. EDTA + benzalkonium chloride shows the repelling effects of surfaces coated with EDTA add to a surfactant, having the lowest amount of *E. faecalis* biovolume ($P < 0.0001$) and viability of cells ($P < 0.0001$) when compared to the other groups. The second lowest values were to EDTA for the former and to EDTA + N-acetylcysteine for the latter. The other groups had no statiscal differences between them for both analysis.

Table II shows the median, minimum and maximum values of smear layer scores before and after the experimental treatments. The Kappa test showed great concordance (0.91) between the examiner at the smear layer evaluation. In the initial analysis (before treatment) there was no difference among the groups ($P > 0.05$). After treatment, all the EDTA-based solutions removed the smear layer with significant differences when compared with the saline solution or sodium hypochlorite ($P < 0.05$).

Table III shows the mean values and standard deviation of surface tension and wettability of the tested solutions by the contact angle measurement (WI). It also shows the wettability values of saline solution after treated dentine with the tested solutions by the contact angle measurement (WD). Lower surface tension and wettability values (WI) were observed to occur in the Groups sodium hypochlorite, EDTA + benzalkonium chloride and EDTA + N-acetylcysteine, which differed statistically from the other groups ($P < 0.05$), except for sodium hypochlorite WI. Significant difference also occurred in the surface tension comparison of Groups EDTA + chlorhexidine, EDTA and saline solution, with decrease values in this order ($P < 0.05$).The groups that favors and harm the wettability of saline solution (WD) in coated dentine were EDTA + benzalkonium chloride and EDTA + chlorhexidine, respectively. No differences were seen among the other groups ($P < 0.005$).

Figure 3 shows the effect of the irrigating solutions at different concentrations on NIH/3T3 fibroblasts viability by MTT assay at 3h of exposure. All the groups exhibited high cytotoxicity at all dilutions tested with significant differences when compared to the control ($P < 0.05$).

Table 1 – Median (Med), minimum and maximum (Min-Max) values of the percentage of live cells (% LC1) and biovolume (BV1) of different biofilms after contact with the experimental solutions for 5 minutes and of the percentage of live cells (% LC2) and biovolume (BV2) of bacteria adherence after 1 hour of contamination on the dentine coated with the experimental solutions for 5 minutes

	EDTA	EDTA/ Benzalkonium Choride	EDTA/ Chlorhexidine	EDTA/ N Acetylcysteine	NaOCl	Saline	No treatment
% LC1	74.86^{A} (38.95 – 95.43)	53.67^{BC} (20.75 – 77.11)	39.27^{ABC} (24.44 – 90.41)	62.97^{BC} (29.05 – 76.96)	49.74^{C} (0.03 – 99.97)	84.50^{A} (42.9 – 96.75)	95.93^{A} (42.90 – 99.92)
BV1	6208^{A} (122.0 – 64105)	64002^{A} (8606 – 669238)	188611^{A} (46042 – 580469)	109650^{A} (8337 – 484023)	6308^{B} (122 – 64105)	158938^{A} (46042 – 484023)	305036^{B} (26877 – 535057)
% LC2	87.57^{AB} (4.40 – 99.94)	0.59^{C} (0.0 – 32.33)	90.4^{A} (49.79 – 100)	72.82^{B} (10.67 – 98.19)	94.06^{AB} (26.9 – 99.92)	91.36^{AB} (19.5 – 100)	
BV2	670.5^{BC} (58 – 4678)	134.5^{C} (1 – 6346)	994.5^{AB} (78 – 7880)	854.5^{AB} (151 – 5162)	1651^{A} (50 – 3426)	804^{AB} (80 – 14626)	

Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in rows indicate statistically significant intergroup differences

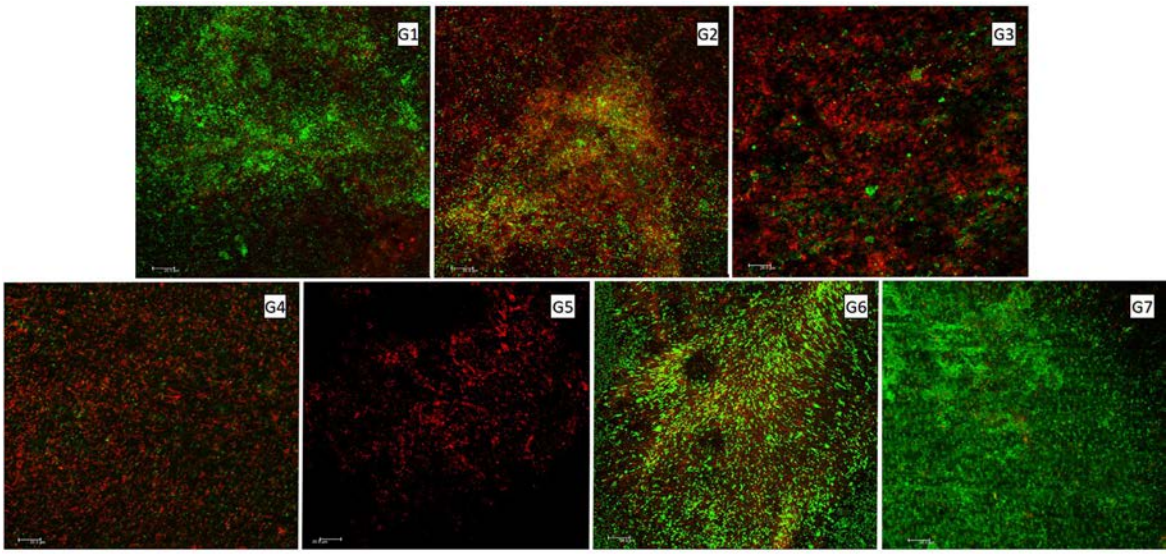

Figure 1 – Confocal laser scanning microscopy of *Enterococcus faecalis* biofilm treated with (G1) 17% EDTA, (G2) 17% EDTA + 1% Benzalkonium Chloride, (G3) 17% EDTA + N-acetylcysteine, (G4) 17% EDTA + Chlorhexidine, (G5) 2.5% NaOCl, (G6) Saline Solution or (G7) no treated. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 x 275 mm

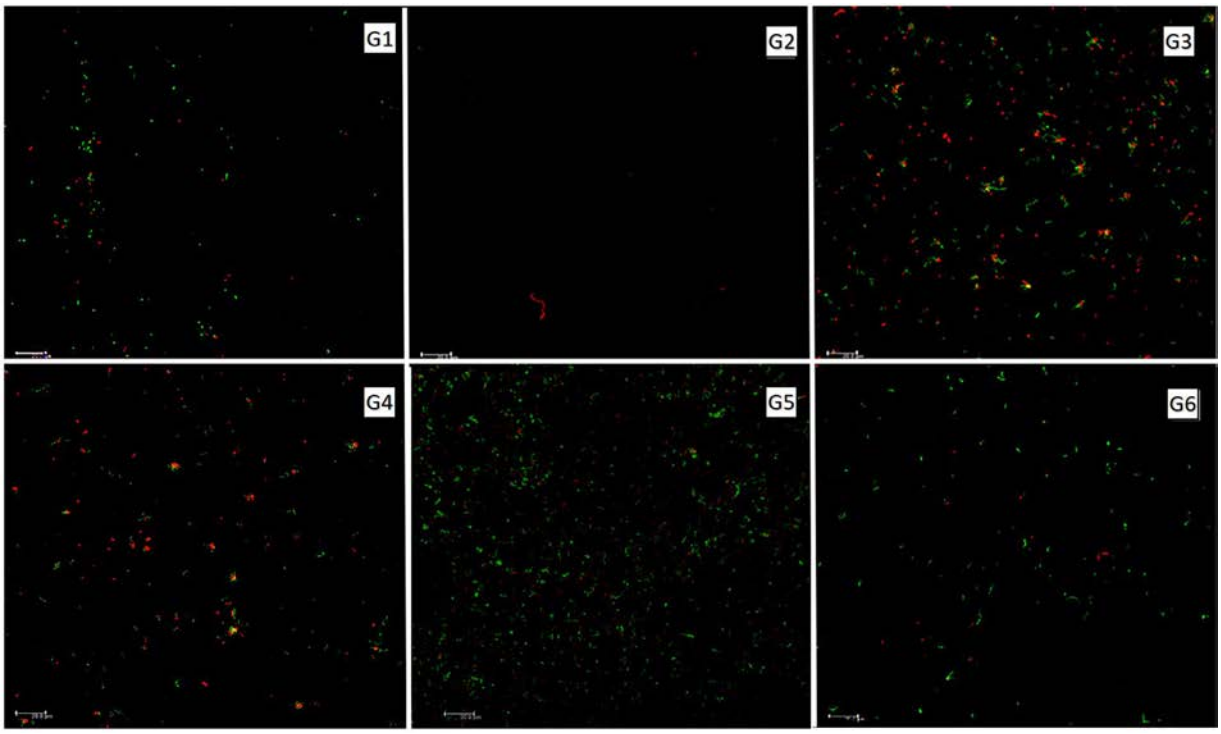

Figure 2 – Confocal laser scanning microscopy of *Enterococcus faecalis* cell adhesion after one hour of contamination on dentine surfaces treated with (G1) 17% EDTA, (G2) 17% EDTA + 1% Benzalkonium Chloride, (G3) 17% EDTA + N-acetylcysteine, (G4) 17% EDTA + Chlorhexidine, (G5) 2.5% NaOCl and (G6) Saline Solution for five minutes. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 x 275 mm

Table II – Median (Med), minimum and maximum (Min-Max) scores for the presence of smear layer in each group before and after 5 min of immersion in the irrigation solutions

EDTA		**EDTA/ Benzalkonium Choride**		**EDTA/ Chlorhexidine**		**EDTA/ N-Acetylcysteine**		**NaOCl**		**Saline**	
T-initial	**T-5min**	**T-initial**	**T-5min**	**T-initial**	**T-5min**	**T-initial**	**T-5min**	**T-initial**	**T-5min**	**T-initial**	**T-5min**
4^{A} (3-4)	2^{B} (1-4)	4^{A} (3-4)	2^{B} (1-4)	4^{A} (3-4)	2^{B} (1-3)	4^{A} (3-4)	2^{B} (1-3)	4^{A} (4-4)	4^{A} (4-4)	4^{A} (4-4)	4^{A} (4-4)

Score 1 – no smear layer, all dentinal tubules open

Score 2 – small amount of smear layer, more than half of the dentinal tubules open

Score 3 – homogenous smear layer covering the root canal wall, less than half of the dentinal tubules open

Score 4 – complete root canal wall covered by a homogeneous smear layer, no open dentinal tubules

Friedman P-value <0.05; different capital letters in rows indicate statistically significant intragroup differences

Table III – Line 1: Mean and standard deviation values of surface tension (ST) for all the irrigants. Line 2: The mean and standard deviation values of the wettability of the irrigants on dentine measured by contact angle measurement (WI). Line 3: Median (Med), Mininum and Maximun (Min-Max) values of surface wettability of dentine after been coated with the irrigant solutions by contact angle measurement (WD)

	EDTA	EDTA/ Benzalkonium Choride	EDTA/ Chlorhexidine	EDTA/ N-Acetylcysteine	NaOCl	Saline
ST	65.52+ 1.87^{B}	34.38+0.46^{D}	68.45+0.98^{A}	35.8+1.53^{D}	29.69+ 1.86^{E}	54.17+ 1.32^{C}
WI	51.10^{A} (39.3 – 63.1)	18.70^{B} (10.4 – 26.2)	53.35^{A} (46.1 – 89.0)	35.70^{B} (7.10 – 42.0)	42.55^{AB} (23.2 – 60.1)	56.30^{A} (25.3 – 83.5)
WD	67.95^{AB} (44.1 – 75.3)	20.30^{C} (7.3 – 32.1)	74.30^{A} (33.8 – 89.7)	39.95^{BC} (10.9 – 66.6)	43.45^{BC} (14.20 – 52.0)	39.10^{BC} (21.7- 49.0)

Line 1: One-way Anova with Tukey post-hoc P value <.05; Line 2: Kruskal-Wallis with a Dunn post hoc P value <.05; Different capital letters in rows indicate statistically significant intergroup differences

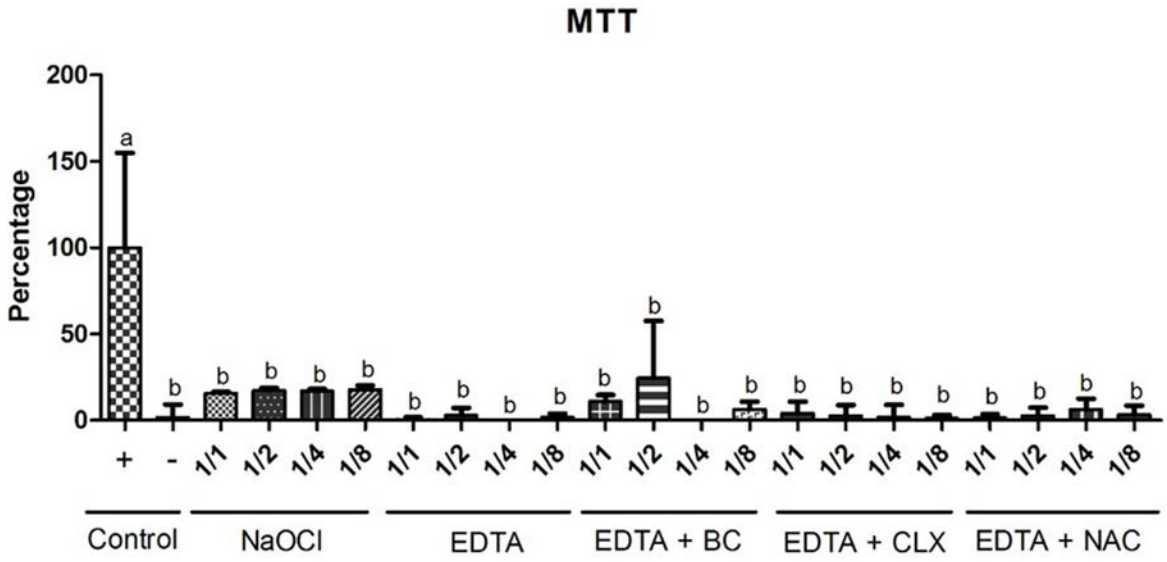

Figure 3 – Effect of the irrigating solutions at different concentrations on NIH/3T3 fibroblasts viability by MTT assay at 3h of exposure. Different letters indicate statistically significant difference (p<0.05)

Discussion

The null hypothesis was rejected because differences were observed in antimicrobial action, adhesion of bacteria to dentine, wettability properties and surface tension of the irrigants when compared to unmodified EDTA.

The commercial form of EDTA (17%), has low antibacterial action, and non-existent antibiofilm action [16, 19], which leads to a final rinse with an antimicrobial agent, such as sodium hypochlorite to optimize disinfection [33, 36]. Sodium hypochlorite is a dangerous chemical if ingested and causes severe bruising when it comes in contact with the soft tissues mainly at high concentrations [4]. Furthermore, it proteolytic action on the collagen matrix of dentine can reduces the elastic modulus and flexural strength of the latter while being used inside the root canal [25]. Using a calcium chelator with antimicrobial properties may reduce the need to use excessive amounts of sodium hypochlorite solution to disinfect the root canal space. This would reduce the harmful effects of the the hypochlorite solution and also reduce treatment time. For this purpose active substances as benzalkonium chloride, n-acetylcisteine and chlorhexidine were added to the EDTA.

The antimicrobial activity of these substances added to EDTA were tested on *Enterococcus faecalis* biofilm. The bacterial communities in the root canal are found as biofilms which are wrapped by an extracellular matrix protected them and increasing their resistance to antimicrobials by about 1000 times when compared to planktonic bacteriam [7]. EDTA affected the membrane integrity in *E. faecalis* after 5 minutes of exposure [5], however, the EDTA concentration used was 50 mmol/L, that corresponds to 38.7% aqueous solution. This high amount of EDTA reduced microhardness, increasing calcium loss, resulting in not only smear layer removal, but erosion of dentine [3]. In agremment with literature [16, 19], 17% EDTA alone did not affect the biofilm having the same behavior as saline solution. As expected, benzalkonium chloride and n-acetylcisteine had superior effect against biofilm than the latter, with no differences from sodium hypochlorite (table I).

The use of a surfactant is important in biofilm disruption since proteins and polysaccharides on the bacterial cell surface attach irreversibly to specific receptors on the substrate (dentine) in a hydrophobic interaction [31]. Surfactants can change this high-affinity binding acting on the surfaces, by either changing bacteria hydrophobicity and bacterial surface charge [26]. This was verified in the current study where the addition of benzalkonium chloride resulted in the lowest amount of *E. faecalis* biovolume (figure 2). This results are in accordance with literature that shows that adding benzalkonium chloride to sodium hypochlorite on dentine promoted inhibition of adhesion, and consequently formation of biofilm [12]. Therefore, EDTA/benzalkonium chloride showed effectiveness not only preventing the formation of biofilm and root canal re-infection, but also against *E. faecalis* biofilm itself when compared to pure EDTA. Chlorhexidine mixture with EDTA forms a white-colored precipitate, formed by neutralization of the cationic chlorhexidine by the anionic EDTA [23]. In this study, the mixture did not have a reduced chelating action when compared with that of pure EDTA, but it might had affect it antimicrobial action [18] and the effect of the adsorption of chlorexidine to dentine [29], no residual antimicrobial effect against *E. faecalis* in it adhesion. Adding a detergent (surface active agent) in this mixture may avoid this, based on a commercial irrigating solution called QMiX (Dentsply Tulsa, Maillefer, Ballaigues, Switzerland) [27]. EDTA is known to expose the dentine collagen. Although studies showed the ability of *E. faecalis* to adhere to collagen [15], this study is in agreement with literature [34] that the adhesion of *E. faecalis* is not increased by the use of EDTA.

The use of sodium hypochlorite and EDTA alternately is necessary for effective dentine cleaning, as the removal of pulpal tissue remnants and the organic components of the smear layer by sodium hypochlorite [6] lead to unexposed orifices of the underlying dentinal tubules (table II). The chelation of calcium ions by EDTA was not negatively influenced by any of the additives tested. The surfactant effect further enhances this action possibly by increasing the penetration of the mixtures into uninstumented areas [1], thereby increasing the disinfection of root canal systems. The low wettability of dentine from previous irrigation with sodium hypochlorite (table III) might also contribute for a better irrigant flow through dentinal tubules. This study, for the first time showed that the addition of the surfactant benzalkonium chloride or the mucolytic agent N-acetylcysteine to EDTA led to a reduction in surface tension of the latter, increasing its wettability on dentine (table III). When EDTA is applied to dentine, the hydroxyapatite is removed (inorganic component) leading to exposure of the collagen fibers, and diminishing the free surface energy [9]. This chemical change in the composition of dentine results in a more hydrophobic surface [2]. This may explain the better measurements of the dentine surface wettability when it was treated with physiological solution in comparison with EDTA. Whereas, a surfactant compound can change the energy of the surface, explaining why the mixture of EDTA with benzalkonium chloride favored the wettability of saline solution in the treated dentine.

Literature divergs when comparing which of the mainly irrigants used during root canal treatment is the most cytotoxic [24, 30, 32]. These differences may be attribute to different cell lines and irrigant dilutions choosen, as well as experimental time. But it agrees with this study on the remarkable cytotoxicity of sodium hypochlorite [30] and EDTA [24] even in the lower dilutions evaluated by MTT assay. Ideally, an irrigation solution is expected to have antimicrobial properties with as low of a toxicity as possible. As shown in the literature and in figure 3, the cytotoxicity of EDTA was not dose-dependent in the dilutions used in both works, having negative effects in the cells even in lower concentrations (2,25%) [24]. EDTA at 1%, and 0.1% [37] or lower concentrations [32] have displayed reduced cytotoxic effects. If future studies shows that these low concentrations does not affect the EDTA smear layer removal, they could be a clinical potentially reality.

The addition of benzalkonium chloride, N-acetylcysteine and chlorhexidine to EDTA resulted in improvement in antibacterial action against *E. faecalis* biofilm, without compromising the EDTA chelating action and cytotoxicity. EDTA + benzalkonium chloride also decreased the adherence of *E. faecalis* to dentin coated with it, lowered EDTA surface tension and allowed better wettability of dentin, standing out from the other solutions. Therefore, the use of this association as a final rinse in endodontic treatment should be further studied.

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