Effect of Ethylenediaminetetraacetic Acid (EDTA) on the Bactericidal Properties of Sodium Hypochlorite Irrigation Solution. An *in vitro* Study

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ABSTRACT

Aim: Sodium hypochlorite (NaOCl) is a potent antimicrobial agent that has the ability to eliminate microbes from root canals. Ethylenediaminetetraacetic Acid **(**EDTA) has been recommended as an adjunctive irrigant in root canal therapy to remove inorganic material from dentinal walls. However, the addition of EDTA to NaOCl causes a potential interaction between the two chemicals resulting in reduced pH, an increase in hypochlorous acid and chlorine gas and subsequent loss of the active hypochlorite ions. The purpose of this study was to determine if addition of EDTA interferes with the bactericidal properties of NaOCl against certain microorganisms found in root canal.

Hypothesis: EDTA, added to NaOCl in a 1:1 ratio, will inhibit the bactericidal properties of NaOCl against root canal microorganisms.

Material and Method: *Enterococcus faecalis* (*E. faecalis*) and *Porphyromonas gingivalis (P. gingivalis)* were chosen for this study and cultured per ATCC recommendations. Bacterial suspensions were incubated for 30s, 3 min or 30 min in Brain Heart Infusion broth (Difco) (negative control group), with 5.25% or 2.625% NaOCl (positive control groups), 17% EDTA, or 5.25% NaOCl mixed with 17% EDTA (1:1 ratio; test group). Total viable counts were determined using the serial dilution and plate counting method. Descriptive statistics (means and standard deviations) were computed. Survival percentages were analyzed via 95% confidence intervals.

Results: The application of NaOCl (5.25%) or NaOCl (2.625%) diluted with H_2O completely eliminated of both *E. faecalis* and *P. gingivalis,* at all-time points tested. EDTA alone did not affect the survival of either bacterium at any time point tested. Importantly, addition of EDTA to NaOCl significantly decreased the bactericidal properties of NaOCl against both bacteria at all time point even after a 30 minute exposure.

Conclusion: Under the limitations of the present study, we concluded that EDTA, used at a 1:1 ratio, interferes with the bactericidal properties of NaOCl.

Key words: Sodium hypochlorite, ethylenediaminetetraacetic acid, *E. faecalis*, *P.gingivalis*, antibacterial effect, root canal therapy, irrigation.

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I. Introduction

A. Structure and function of the dentin-pulp complex

The dental pulp is a sterile organ that plays an important role in the formation of dentin and tooth sensation. The dentin is a mineralized tissue that is protected from exogenous substances in the oral cavity by enamel in the crown and cementum on the root surface (1). The presence of dentinal tubules in the dentin allow microorganisms to adhere to the tissue surface, and enter the pulp (1). Openings in the enamel and/or dentinal wall resulting from caries, clinical procedures, or trauma-induced fractures and cracks can lead to pulp-dentin complex infection (2-4).

B. Microbiology and pathobiology of the root canal and periapex

When the dental pulp is infected with microorganisms, pulp tissue undergoes inflammation with subsequent necrosis. The necrotic pulp becomes a reservoir for infection and possibly become isolated from the patient's immune response. Eventually, bacteria and bacterial by-products will evoke a periradicular inflammatory response (5, 6). The diffusion of bacterial metabolites and toxins into the periapical tissues leads to inflammatory diseases, e.g. apical periodontitis, which can be characterized by resorption of alveolar bone (1).

One of the well-established factors in the development of periradicular disease is the presence of bacteria and their by-products. A study by Kakehashi *et al*. (6) exposed the dental pulps of conventional rats and germ-free rats by creating an opening in the occlusal surface of the maxillary right first molars. The result reported that bacteria are the main factor for the development of pulp necrosis and periradicular lesions.

More than 300 bacterial species are recognized as components of the oral microbiota (7). However, only relatively few bacterial species appear to be able to adhere and invade the

root canal space and cause root canal infection (8, 9). The presence of several distinct types of microorganisms in the necrotic dental pulp was demonstrated more than a century ago (10).

Root canal infections are polymicrobial infections, characterized by mostly anaerobic bacteria and some facultative bacteria (11). Infected dentin is commonly found in primary root canal infections as well as infection of a previously treated tooth (12).

In the primary infected canal with apical periodontitis, the ecological selection in the canal favors strictly anaerobic bacteria. The infection may be purely anaerobic, but microaerophilic and facultative bacteria such as *Actinomyces spp*., *Lactobacillus spp*., and *streptococci* may be present in primary infection (13-15).

In previously root-filled teeth with apical periodontitis, the ecology may be quite different, and in many cases the environment may no longer support the dominance of anaerobic bacteria (16). The most dominant isolated species by far in previously root-filled teeth with apical periodontitis is *Enterococcus faecalis (E. faecalis)*, but several other facultative and even anaerobic bacteria are often isolated (17-20). While mono infections are not detected in the primary infected canal, *E. faecalis* is often found in pure culture in previously root-filled teeth with apical periodontitis. Gram-negative enteric rods (e.g. *Coliform* and *Pseudomonas spp*.) and yeasts are found almost entirely only in previously root-filled teeth with apical periodontitis (17-20).

According to a study by Sundqvist et al., *E. faecalis* had been isolated from 38% of infected teeth (21). Furthermore, Molander *et al.* (17) examined the microbiological status of root filled teeth with apical periodontitis and found that facultative anaerobes predominated with *enterococci* being the most frequently isolated group. Hancock *et al*. (19) examined root

filled teeth with persistent periapical radiolucencies, and found that as well as *Enterococcus*, other genera, *Peptostreptococcus*, *Actinomyces*, and *Streptococcus*, are predominant. Additionally, *Enterococcus* has been isolated from 47% of root canals in which treatment had failed (22).

Generally, facultative anaerobes are more resistant to anti-microbial agents than are obligate anaerobes and are therefore more likely to survive root canal treatment unless cleaning and shaping procedures are of the highest standard (23, 24). *Enterococci* are one of the microorganisms that have the ability to live in a harsh environment. It has been recognized to persist during root canal treatment (25), because of its ability to grow at high pH (26). It also can hold off certain chemical agents, including some antibiotics, which would be very harmful to other organisms (27). Additionally, it cannot be killed with intracanal medicaments such as calcium hydroxide because of its ability to release protons that equalize the high pH of calcium hydroxide (28).

C. Nonsurgical root canal treatment

a. Chemomechanical preparation

Endodontic treatment helps in preventing or treating an existing apical periodontitis and irreversible pulpitis (27). Instrumentation and irrigation techniques remove necrotic debris and vital organic tissue as well as some hard tissue from the root canal system, and gives the canal system a shape that allows the placement of medicaments and a permanent root filling. It also helps in reducing microorganisms in the root canal system, and neutralizes any antigenic/ biological potential of the microbial components remaining in the canal (16). Therefore, mechanical instrumentation is the main method in root canal therapy for bacterial elimination (28). However, the complexity of the root canal anatomy as well as the multiple

fins and ramifications make root canal instrumentation effective in cleaning only 65% of the root canal surface, leaving 35% of the canal surface area untouched (29).

b. Root canal irrigation

The use of irrigating solutions is an important part of effective chemomechanical preparation. Root canal irrigants are used to kill bacteria, dissolve necrotic pulp tissue, inactivate endotoxin, and prevent the formation of a smear layer (30).

1. Sodium hypochlorite

Sodium hypochlorite (NaOCl) is the most widely used irrigation solution in endodontic practice (31). It is the main endodontic irrigant and was recommended in endodontic practice by Coolidge (32). Commercial sodium hypochlorite solutions are strongly alkaline, hypertonic, and typically have nominal concentrations of 10 to 14 percent available chlorine.

Sodium hypochlorite is a potent antimicrobial agent, and effectively dissolves pulpal remnants and organic components of dentine. It is used both as an unbuffered solution at pH 11 and buffered with bicarbonate at pH 9.0. Sodium hypochlorite deteriorates with time, temperature, exposure to light, and contamination with metallic ions. Sodium hypochlorite exhibits a dynamic balance as shown by the following reaction (33):

$$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

Sodium hypochlorite is used in endodontic practice in concentrations varying from 0.5% to 5.25%. Gomes *et al*. tested the effect of various concentrations of sodium hypochlorite in vitro against *E. faecalis*, and showed that 5.25% NaOCl removed all microbes in less than 30 s, while it took 10 and 30 min for complete killing of the bacteria by 2.5% and 0.5% of NaOCl, respectively (27).

Sodium hypochlorite has a tissue-dissolving effect on necrotic and vital pulp tissue, and a wide spectrum of nonspecific killing efficacy on all microbes (34, 35). Moreover, sodium hypochlorite is efficient in mechanically flushing the debris from root canals. McComb and Smith (36) reported that when canals were irrigated with 6% sodium hypochlorite, it resulted in almost sterile canals.

Chlorine in aqueous solution at body temperature can take two forms: hypochlorite (OCl-) or hypochlorous acid (HOCl), both of which refer to active chlorine content. Both forms are extremely reactive oxidizing agents (37). Chlorine possesses antimicrobial action by inhibiting bacterial enzymes leading to an irreversible oxidation of sulfhydryl (SH) groups (33). The antimicrobial effectiveness of sodium hypochlorite is based on its high pH (hydroxyl ions action). Estrela *et al* reported that sodium hypochlorite interferes with cytoplasmic membrane integrity, alters the cellular metabolism and degrades the phospholipid membrane (33). Thus, sodium hypochlorite effectively eliminates microbes from root canals (34, 38) and also kills bacteria within open dentinal tubules (39).

Although sodium hypochlorite appears to be the most desirable single endodontic irrigant, it has been criticized for its unpleasant taste, relative toxicity, and its inability to remove the smear layer (40).

2. Smear Layer

The smear layer is a thin layer of mineral salts and organic debris that occlude the dentinal tubules and covers the intertubular dentin of the canal wall (41). The smear material consist of two parts; the superficial smear layer, which is about 1 to 2 μm in thickness, and the deep layer which is packed into the dentinal tubules. Packing of smear debris was present in the tubules to a depth of 40 μm (42) (43).

The smear layer contains organic and inorganic substances that include fragments of odontoblastic processes, microorganisms and necrotic materials (44). This layer is considered deleterious because it prevents the penetration of disinfectant agents and medications, and it impedes the adherence of sealing material to the dentinal tubules (43, 45, 46).

3. Ethylenediaminetetraacetic acid

A demineralizing agent such as ethylenediaminetetraacetic acid (EDTA) has been recommended as an adjunctive irrigant in root canal therapy (47). Ostby (48) proposed that EDTA could substitute inorganic acid in root canal therapy because it had certain dentin-dissolving effects desirable in all kinds of root canal therapy.

EDTA removes mineral salts from obstructed dentin by chelation and leaves only the softened matrix. Saito *et al*. (49) reported that root canal irrigation with 17% EDTA for 1 min was more effective than in 30 s in removing the smear layer after root canal instrumentation. It has also been reported that EDTA decalcified dentin to a depth of 20-30 μm in 5 min (50). However, according to Patterson (51), EDTA has limited antibacterial activity. It seems that the antibacterial activity of EDTA is due to the chelation of cations from the outer membrane of bacteria. Arias-Moliz *et al*. (52) showed that EDTA had no effect against *E. faecalis* even after 60 min contact.

4. Interaction of NaOCl with EDTA

The use of both chemicals, NaOCl and EDTA, during root canal preparation to produce the cleanest canal walls is recommended (30, 53). Several authors have agreed that the removal of the smear layer as well as soft tissue and debris can be accomplished by the alternate use of EDTA and NaOCl (54-57). Goldman *et al*. (58) examined the effect of

various combinations of EDTA and NaOCl, and the most effective final rinse was found to be 10 mL of 17% EDTA followed by 10 mL of 5.25% NaOCl.

However, the addition of chelators like EDTA to NaOCl causes a potential interaction between the two chemicals; it reduces the pH in a ratio and time-dependent manner, increases hypochlorous acid and chlorine gas and subsequent loss of the active hypochlorite ion (35, 40, 53, 59). The dramatic reduction in active chlorine content may explain the inability of sodium hypochlorite to eradicate bacteria and dissolve soft tissue (60, 61).

Irala *et al*. (60) evaluated the tissue dissolving ability of NaOCl (1-2.5%) alone and combined with 17% EDTA in different ratios (2:2 and 1:3). Findings indicated that after 48 hrs, only unmixed NaOCl was able to completely dissolve the tissue. Grawehr *et al*. (62) confirmed the findings of Irala *et al*. Saquy *et al.* (63) reported that the addition of NaOCl to EDTA did not alter EDTA's ability to decalcify human dentin. NaOCl does not reduce the calcium chelating or the smear layer removal ability of EDTA and citric acid (59).

However, the effect of EDTA on the bactericidal properties of NaOCl has not been thoroughly tested so far. Therefore, the experiments described in this study were designed in order to test if adding EDTA to NaOCl diminishes or inhibits its antibacterial properties.

II. Objective

The purpose of this study was to evaluate the antibacterial properties of solutions consisting of NaOCl mixed with either distilled water or EDTA on two root canal microorganisms. This was accomplished by analyzing bacterial survival after incubation in NaOCl solutions mixed with or without EDTA.

III. Hypothesis

EDTA, added in a 1:1 ratio, will inhibit the bactericidal properties of NaOCl against root canal microorganisms.

IV. Materials and Methods

The microorganisms used in this study were *Enterococcus faecalis* (ATCC 29212), and *Porphyromonas gingivalis* (ATCC 33277) (Table 1). These microorganisms were selected based on their presence in a higher percentage of primary infected root canals and in previously root canal treated teeth (2).

Five test conditions were used (Table 2). G1, which received brain heart infusion broth acted as negative control group and G4 received EDTA. The other three conditions were prepared from a commercially available 5.25% NaOCl stock solution (Clorox, Oakland CA) and used undiluted (G2), or diluted, at 1:1 ratios, with distilled water (G3) or 17% EDTA solution (G5, Endo-cleanse, Roydent, Johnson City, TN) (Table 2). Solutions were prepared immediately before use and maintained at room temperature. Based on both the known bactericidal properties of NaOCl and results from a pilot study (preliminary analysis), we expected 100% survival in G1 and 100% killing in G2 and G3. These groups, as well as G4, were used solely as intra-experimental controls and therefore were not subjected to statistical analysis.

Broth and agar plates were prepared 2-5 days before the experiment and stored at 4°C. To prepare *E. faecalis* broth and agar plates, either 52 g of brain heart infusion agar (BD 211065) or 37 g of brain heart infusion broth (BD 237500) were mixed with 1L of distilled water, respectively. The solutions were autoclaved at 121°C, then stored at 4°C. For *P. gingivalis* strain, broth was prepared by mixing 18.5 g of Brain heart infusion broth (BD 237500) and 5 g yeast extract with 1L of distilled water and autoclaved at 121°C. Once the mixture cooled, the following ingredients were added: 10 μg/ml hemin, 1 μg/ml

menadione/vitamin K, 1 g sodium bicarbonate, 250 mg sodium thioglycolate, and 500 mg cysteine. Then the broth was filter sterilized and stored at 4°C. Blood agar plates for *P. gingivalis* were prepared by Tufts University Molecular Biology and Microbiology Media Service. To mimic the clinical situation, laboratory work was performed in aerobic conditions while handling the bacteria anaerobically as much as possible.

Fresh broth cultures of each bacterium were prepared by inoculating 5 mL of the appropriate medium in an 18x150 mm test tube with either a single colony from an agar plate or 100 µl of a previous culture. Cultures were incubated at 37°C in either aerobic or anaerobic conditions. The incubation period was 24hrs for *E. Faecalis* and 6 days for *P. gingivalis*. The growth rate of each microorganism was recorded using a standard curve that relates turbidity (measured spectrophotometrically) to colony forming units (CFU) (data not shown). These tubes constituted the primary cultures.

In each tube, 950 µL of the test solution was mixed with 50 µL of the bacterial suspension for 30 seconds, 3 minutes or 30 minutes (Figure 1). An inactivator (5% sodium thiosulfate) was added to the groups that contained NaOCl to reduce any carryover effect, as described by Pappen *et al*. (64). Samples were then removed and viable counts determined as described below.

Total viable counts were determined using the serial dilution and plate counting method. For each bacterium, seven sterile tubes were labeled with 10^1 to 10^7 of the dilution factors. Seven agar plates were similarly labeled with the dilution factors 10^1 to 10^7. Using aseptic techniques, 900 µl of the broth were added to each tube. Then 100 µl of bacterial culture were added to the tube labeled 10^1 and mixed by pipetting up and down

five times. One hundred µl of the diluted cell culture was then removed from tube labeled 10 ^1 and added to the tube labeled 10 ^2 and mixed by pipetting up and down five times. Dilutions continued with bacterial culture in each consecutive tube until the tube labeled 10 ^7. One hundred µl aliquots of each dilution tube were spread over the entire surface of the corresponding agar plate and the plates incubated at 37°C aerobically for 24hrs for *E. faecalis* and anaerobically for 6 days for *P. gingivalis*. The number of colonies per plate was determined once the colonies were large enough to count.

V. Statistical Analyses

Sample size calculation

A sample size calculation was conducted using nQuery Advisor (Version 7.0). Assuming a within-group standard deviation of 0.127% for the outcome of survival percentage*, a sample size of n = 6 was deemed adequate to have a 95% probability of obtaining a 95% confidence interval with a margin of error no more than 0.2%.

* Result obtained from a pilot study

Data presentation and statistical analyses

Descriptive statistics (means and standard deviations) were computed. Survival percentages were analyzed via 95% confidence intervals. All analyses, including initial descriptive statistics, were calculated using SPSS Version 22.

VI. Results

E. faecalis cultures were treated with undiluted NaOCl, NaOCl diluted 1:1 with H_2O, EDTA, or NaOCl mixed 1:1 with EDTA for 30 seconds, 3 and 30 minutes. The number of surviving bacteria was determined using the serial dilution and plating technique. Undiluted NaOCl or NaOCl diluted 1:1 with H_2O resulted in complete killing of the bacteria. In contrast, cultures incubated with EDTA alone or more importantly when EDTA was mixed with NaOCl, still contained viable bacteria. The data from 6 experiments were analyzed and are presented in Table 3 as means (95% confidence intervals) and in Figure 2 as means (SD). In general, the data were generated using the 10^5 dilution samples but similar data were obtained when the 10^6 dilution was used (data not shown).

In the undiluted NaOCl group or the NaOCl diluted with H_2O group, there was no bacterial survival at all-time points tested (data not shown). The bactericidal effect of undiluted NaOCl or NaOCl diluted with H_2O was observed even at the low dilution samples, i.e., 10^1 (data not shown).The mean percent survival (SD) for the EDTA group was 63.82 (21.73), 64.68 (7.82), and 59.82 (10.94) for the 30 s, 3 min, and 30 min incubation periods, respectively. When cultures were incubated in the presence of NaOCl diluted 1:1 with EDTA, percent bacterial survival means (SD) were 12.28 (4.03), 11.05 (1.74), and 11.93 (2.57) for the 30 sec, 3 min, and 30 min incubation periods, respectively. The fact that the percent survival did not decrease with increased time of incubation (3 and 30 min) suggests that active hypochlorite products were no more available past the 30 s incubation time in the presence of EDTA. Therefore, it was concluded that the addition of EDTA inhibited the bactericidal properties of NaOCl against *E. faecalis.*

Similar findings were obtained when *P. gingivalis* was used. As with *E. faecalis*, when cultures of *P. gingivalis* were incubated in the presence of undiluted NaOCl or NaOCl diluted with water, no bacteria survived at all time periods tested. In contrast, viable cells could be recovered in cultures incubated with EDTA or NaOCl mixed 1:1 with EDTA. Table 4 (means and 95% confidence intervals) and Figure 3 (means and SD) show the data analyzed from 6 experiments using the 10^5 dilution samples. Similar data were obtained when the 10^6 dilution was used (data not shown).

In the undiluted NaOCl group or the NaOCl diluted with H_2O group, no bacteria survived at all incubation periods tested (data not shown). Again, the bactericidal effect of undiluted NaOCl or NaOCl diluted with H_2O was observed even at the low dilution samples, i.e., 10^1 (data not shown). The mean percent survival (SD) for *P. gingivalis* incubated in the presence of EDTA for 30 s, 3 min, and 30 min, were 68.62 (4.84), 57.10 (7.70), and 59.72 (9.51), respectively. When cultures were incubated in the presence of NaOCl diluted 1:1 with EDTA, percent *P. gingivalis* survival mean (SD) were 38.22 (3.02), 27.08 (5.57), and 28.87 (6.96) for the 30 sec, 3 min, and 30 min incubation periods, respectively. The fact that the percent survival did not decrease with increased time of incubation suggests that active hypochlorite products were no more available past the 30 s incubation time in the presence of EDTA. It was concluded that, as for *E. faecalis*, the addition of EDTA inhibited the bactericidal properties of NaOCl against *P. gingivali*.

Taken together, the data implies that addition of EDTA, in 1:1 ratio, to NaOCl solutions significantly decreased its bactericidal properties against two bacteria that are commonly found in root canals.

VII. Discussion

Previous studies reported the need for using different endodontic irrigation solutions during chemomechanical preparation to achieve a thoroughly cleaned root canal (42, 54, 65, 66). Sodium hypochlorite is the most common irrigant used in root canal therapy. This solution has been known for its excellent antimicrobial and tissue-dissolving actions. The exact mechanism of the microbial killing and tissue dissolving effects are unknown. However, it is most likely the function of the free available chlorine (OCl- /HOCl) (67). Additionally, In 1957, Ostby (48) advocated the use of a chelating agents such as 15% EDTA as an irrigant during endodontic therapy to chelate inorganic material (smear layer) that resulted from the mechanical preparation. Cobankara *et al*. reported in a recent study the importance of the removal of the smear layer since it negatively influences the coronal and apical seal of endodontically obturated teeth (68).

Adding a chelating agent to NaOCl will reduce the pH in a ratio and time dependent manner (40, 53, 60). A laboratory investigation by Zehnder (40) showed that mixing 1% NaOCl (pH = 12.12) with 17% EDTA (pH = 7.51) in 1:1,1:5, and 5:1 ratio resulted in a reduction of the total pH solution to 8.0. This interaction will affect the free chlorine in the solution and elevate the level of the hypochlorous acid and chlorine gas (69, 70). Subsequently this may affect the antimicrobial action and the tissue dissolving properties of the NaOCl solution. Moreover, the study of Clarkson *et al*. showed the dramatic reduction in the active chlorine content of NaOCl when it is mixed with EDTA in different ratios. Eight percent of the active chlorine content was lost if NaOCl was mixed with EDTA at 90:10 ratio for 5 min. This percentage increased up to 36% in 75:25 ratio, and further reduction was seen once the 50:50 ratio was reached (35). It was concluded that even 10 % dilution of NaOCl

with EDTA may affect the activity of the NaOCl solution. Additionally, Baumgartner's study measured the chlorine gas evaporation and the presence of an exothermic reaction when EDTA was mixed with NaOCl. This confirmed the presence of a chemical interaction between the two solutions which may affect the function of NaOCl (53). An investigation by Irala et al. (60) studied the dissolving effect of different concentrations of NaOCl (1% - 2.5%) alone or when mixed with 17% EDTA in different ratio (2:2 and 1:3), on bovine tissue for 48 hours. Their findings showed that only unmixed NaOCl was able to dissolve the bovine tissue completely (60).

To date, the effect of EDTA on the antibacterial properties of NaOCl have not been extensively studied. Therefore, this study was designed to evaluate the antimicrobial action of 5.25% NaOCl when mixed with 17% EDTA using two types of bacteria and three incubation periods. This study was performed using only two strains of bacteria; this does not exactly mimic the typical clinical situation. Endodontic infection is a mixture of necrotic tissue with a polymicrobial flora and smear layer. *E. faecalis* and *P. gingivalis* were chosen, as they have been shown to be some of the most common pathogens present in endodontic infections. *E. faecalis* is frequently recovered in failed endodontic treatment (25, 71) and has the ability to resist several endodontic medicaments such as calcium hydroxide (72, 73). This bacterium not only has the ability to reside in the canal as single species without the support of another bacterium (14), but has the ability to penetrate deeply inside the dentinal tubules. A study by Haapasolo and Orstavik (74) in a bovine model showed the ability of *E. faecalis* to invade dental tubules up to 300-400 μm after 1 day, and up to 800 μm into the canal after 3 weeks. Another study by the same group (75) showed that for this bacterium, it takes only 2 days for the whole length of the canal to get infected.

P. gingivalis is a black-pigmented gram-negative anaerobic bacterium that plays an important role in endodontic infection. The presence of *P. gingivalis* is usually accompanied by swelling and pain with percussion and palpation sensitivity (11, 76, 77). Gomes *et al.* (78) reported that *P. gingivalis* was the most frequently isolated species in necrotic pulp. It accounted for 38% using polymerase chain reaction technique (PCR). Another study by Tomazinho *et al.* reported the prevalence of *P. gingivalis* in endodontic infection using culture and PCR technique. *P. gingivalis* was identified by culture 27.3% of the time and by PCR 43.3% (79).

The major finding from the present study was that mixing NaOCl with EDTA inhibited the bactericidal ability of NaOCl. The results of this study are in agreement with a previous investigation by Grawehr et al. (62). They analyzed the antimicrobial action of 1% NaOCl mixed with 17% EDTA in 1:1 ratio using agar diffusion test against *E. faecalis* and *C. albicans*. Undiluted NaOCl showed a significant zone of inhibition compared to pure EDTA or a mixture of NaOCl and EDTA. Another in vitro study by Zehnder et.al (40) also reported an inhibitory effect of 10% citric acid or 17% EDTA on the antimicrobial properties of NaOCl (1%) against *E. faecalis*. However, in both studies a lower concentration of NaOCl (1%) was used compared to the more commonly clinically used 5.25%. EDTA inhibited the antibacterial of NaOCl even at this highest concentration.

Our data clearly indicate that mixing of NaOCl and EDTA during root canal instrumentation should be contraindicated. However the order in which the two solutions are used is still debatable. Zehnder (30) advocated the use of NaOCl solution during the entire root canal instrumentation step followed by EDTA used separately for a brief period of time, then a chlorhexidine solution if needed. Other studies had recommend the use of EDTA

during preparation as lubricant to avoid instrument complication such as separation (80, 81). In a study by Nguy and Sedgley, stratification of the solutions occurred throughout the entire length of the root canal if NaOCl and EDTA were used alternatively. Thus the anticipated therapeutic action of the irrigant may not be realized if one of the solutions is not completely eliminated before the use of the other (82). If both solutions are used alternatively, emptying of the canal using a fine cannula to aspirate solution in the apical extremity or vigorously drying the root canal is recommended to obtain the maximum benefit of each irrigant (83). Additionally Zehnder et.al (40) suggested rinsing the canal with copious amounts of NaOCl to make sure the fluid exchange occurs at all levels of the root canal and to prevent stratification.

Sodium hypochlorite does not affect the chelation ability of EDTA (59). A study by Saquy et al. examined the chelation ability of 17% EDTA when mixed with either distilled water or 0.5% NaOCl using a standardized dentin disks. The results indicated that greater calcium chelation occurred in the solution containing NaOCl (63). Thus, chelation agents can affect the antimicrobial activity of NaOCl solutions while the NaOCl did not affect the performance of EDTA (40, 62).

VII. Clinical Relevance

This study showed that mixing 5.25% NaOCl with 17% EDTA in a 1:1 ratio significantly reduced the antibacterial properties of NaOCl. Therefore, caution should be taken when these two irrigants are used simultaneously during chemomechanical instrumentation. Confirming the removal of EDTA with different methods before the use of NaOCl may prevent alterations in the antimicrobial action of NaOCl.

VIII. Limitations of the Study

1- This study was conducted to evaluate the antimicrobial action of NaOCl against two separate microbial strains, which did not mimic the usual polymicrobial status clinically.

2- This study was conducted in test tubes, which did not simulate teeth where microorganisms can travel deep inside the dentinal tubules and other anatomic anomalies and may not be reachable by antimicrobial irrigants.

3- This study used the combination of NaOCl and EDTA in 1:1 ratio. Using other ratios may give different results.

X. Conclusions

Under the limitation of the present study, we concluded that EDTA, used at a 1:1 ratio, interfered with the bactericidal properties of NaOCl.

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APPENDICES

Appendix A: Tables

Appendix B: Figures

Appendix C: Raw data

Appendix A: Tables

Table 1. List of the bacteria strains and growth environment

Bacterium	Medium	Growth conditions
Enterococcus faecalis **(ATCC 29212)**	ATCC Medium 44: Brain Heart Infusion Agar/ Broth	Temperature: 37°C Atmosphere: Aerobic
Porphyromonas gingivalis **(ATCC 33277)**	ATCC Medium 2722: Brain Heart Infusion Broth/ Blood agar	Temperature: 37°C Atmosphere: Anaerobic gas mixture, 80% N_2-10%CO_2-10%H_2

Table 2. List of the conditions in volumetric proportions

Group	Solutions	Solution's Volume	Final concentration of NaOCl
G1	BHI Broth	-	0%
G2	5.25%NaOCl	1mL	5.25%
G3	5.25%NaOCl + Distilled water	1mL (500 µL NaOCl + 500 µL distilled water)	2.62%
G4	17% EDTA	1mL	17%
G5	5.25% NaOCl + 17% EDTA	1mL (500 µL NaOCl + 500 µL EDTA)	2.62% NaOCl + 8.5% EDTA

Table 3. Percent survival of *E. faecalis* in the presence of EDTA or NaOCl mixed 1:1 with EDTA. Data are presented as means (95% confidence intervals), n=6.

	EDTA	**NaOCl + EDTA**
30 sec	63.82 (41.01-86.62)	12.28 (8.05-16.52)
3 min	64.68 (56.47-72.89)	11.05 (9.23-12.87)
30 min	59.82 (48.34-71.30)	11.93 (9.23-14-63)

Table 4. Percent survival of *P. gingivalis* in the presence of EDTA or NaOCl mixed 1:1 with EDTA. Data are presented as means (95% confidence intervals), n=6.

	EDTA	**NaOCl + EDTA**
30 sec	68.62 (63.54-73.70)	38.22 (35.05-41.39)
3 min	57.10 (49.02-65.18)	27.08 (21.24-32-93)
30 min	59.72 (49.73-69.70)	28.87 (21.56-36.17)

Appendix B: Figures

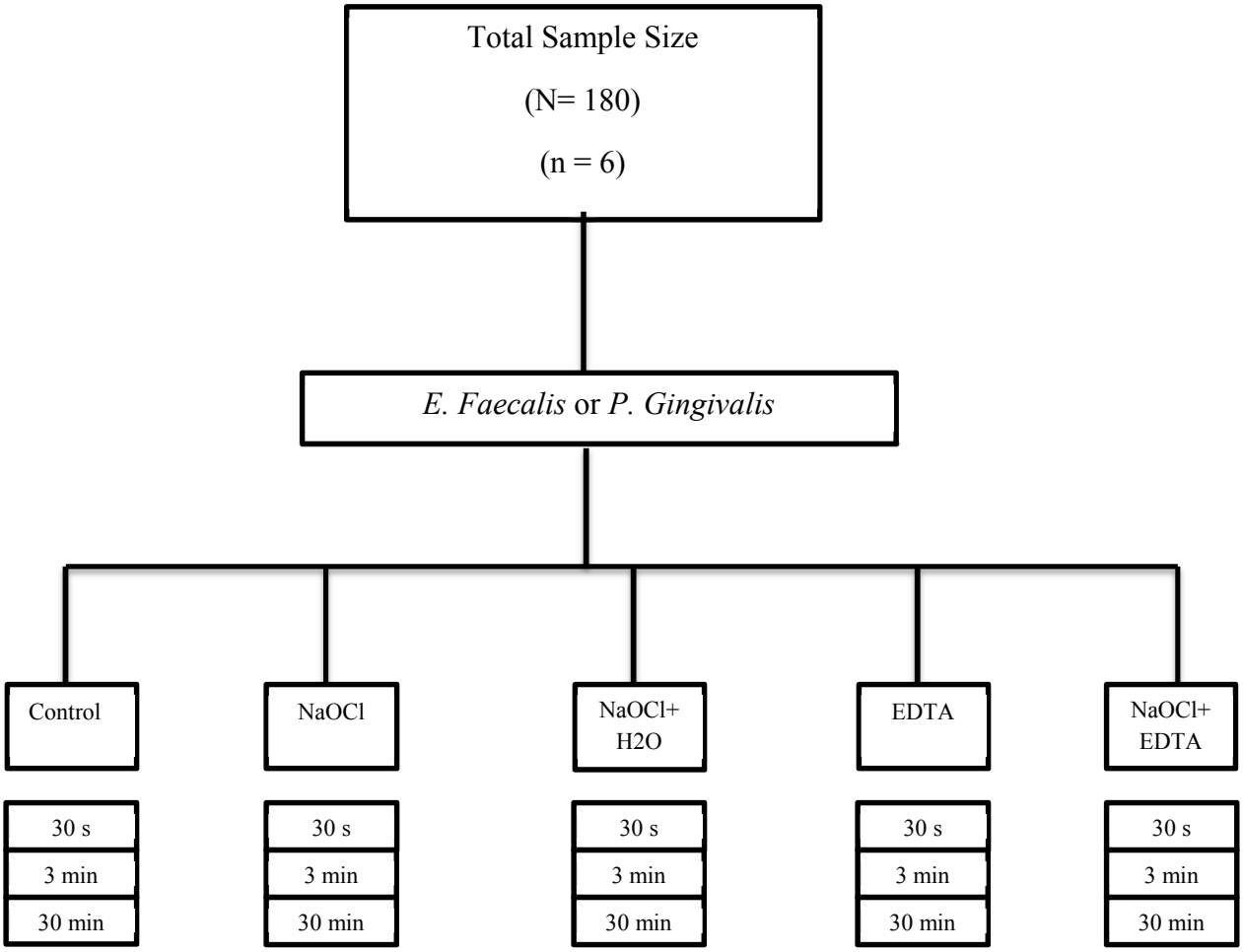

Figure 1. Flowchart depicting the experimental groups and conditions.

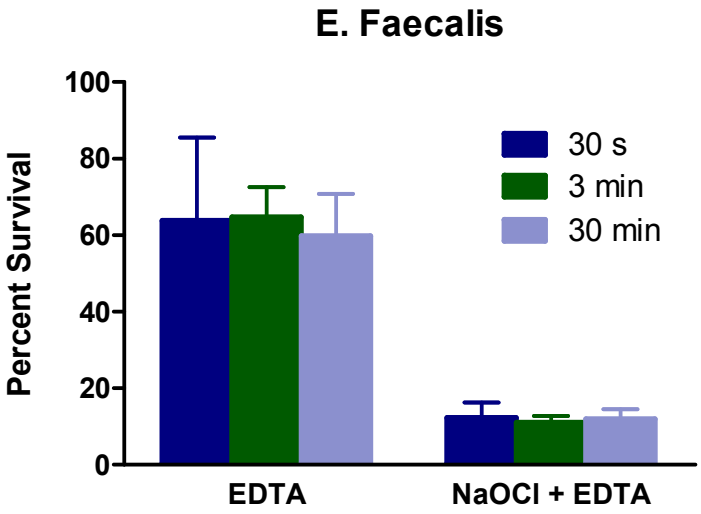

Figure 2. Effect of EDTA and NaOCl mixed with EDTA on *E. faecalis* survival. Data are means ± SD, n=6.

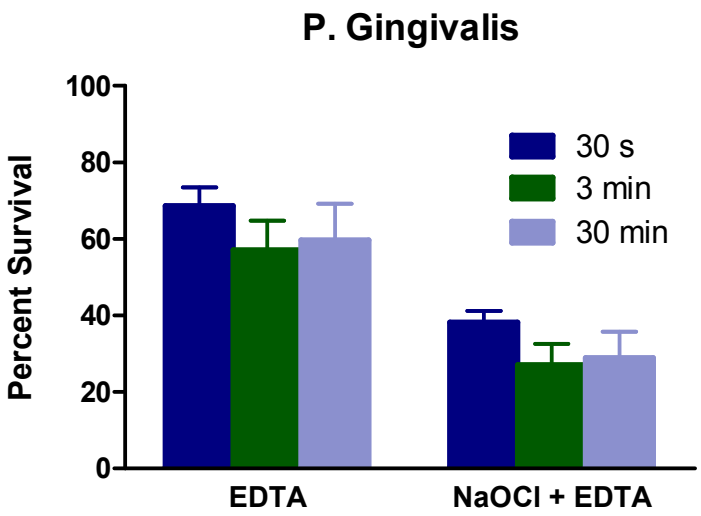

Figure 3. Effect of EDTA and NaOCl mixed with EDTA on *P. gingivalis* survival. Data are means ± SD, n=6.

Appendix C: Raw data

Number of E. faecalis colonies counted in 6 experiments						
Experiment 1						
	30 seconds		3 minutes		30 minutes	
Dilution	5	6	5	6	5	6
Broth	200	14	216	17	250	30
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	98	7	139	10	170	23
NaOCl+ EDTA	37	3	25	6	29	9
Experiment 2						
Dilution	5	6	5	6	5	6
Broth	260	36	320	44	340	52
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	204	18	210	27	232	32
NaOCl+ EDTA	29	4	34	4	33	9
Experiment 3						
Dilution	5	6	5	6	5	6
Broth	355	45	366	46	500	63
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	193	23	270	21	275	24
NaOCl+ EDTA	40	5	43	6	54	7
Experiment 4						
Dilution	5	6	5	6	5	6
Broth	218	41	326	44	331	55
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	214	21	230	30	241	33
NaOCl+ EDTA	33	7	43	5	56	8
Experiment 5						
Dilution	5	6	5	6	5	6
Broth	503	49	522	55	602	71
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	206	17	287	25	287	16
NaOCl+ EDTA	44	7	53	7	73	10
Experiment 6						
Dilution	5	6	5	6	5	6
Broth	350	33	450	45	563	70
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	222	20	273	17	281	19
NaOCl+ EDTA	29	4	38	3	59	8

Number of P. gingivalis colonies counted in 6 experiments						
Experinment 1						
	30 seconds		3 minutes		30 minutes	
Dilution	5	6	5	6	5	6
Broth	101	20	185	15	111	14
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	74	19	97	17	77	9
NaOCl+ EDTA	40	4	41	4	44	8
Experinment 2						
Dilution	5	6	5	6	5	6
Broth	100	20	204	30	203	24
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	77	18	114	22	104	14
NaOCl+ EDTA	39	9	59	9	51	9
Experinment 3						
Dilution	5	6	5	6	5	6
Broth	107	27	207	37	144	22
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	64	12	102	20	98	13
NaOCl+ EDTA	41	7	45	9	47	7
Experinment 4						
Dilution	5	6	5	6	5	6
Broth	132	22	163	26	258	37
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	93	13	91	19	133	21
NaOCl+ EDTA	43	7	41	8	55	10
Experinment 5						
Dilution	5	6	5	6	5	6
Broth	118	28	111	21	183	28
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	80	15	80	11	120	19
NaOCl+ EDTA	40	10	41	5	41	7
Experinment 6						
Dilution	5	6	5	6	5	6
Broth	100	20	205	35	201	30
NaOCl	0	0	0	0	0	0
NaOCl+ H2O	0	0	0	0	0	0
EDTA	68	13	104	14	99	19
NaOCl+ EDTA	40	4	50	5	59	9