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Research Article

Comparison of the Antimicrobial Effect of Chlorine Dioxide, Sodium Hypochlorite and Chlorhexidine, on Bacteria Isolated from the Root Canal

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Abstract

Aim: To compare the antimicrobial capacity of Chlorine Dioxide (ClO_2), Sodium Hypochlorite (NaOCl) and Chlorhexidine (CHX) in microorganisms isolated from persistent apical periodontitis and ATCC strains.

Materials and methods: The microorganisms included were analyzed by Minimum Inhibitory Concentration (MIC): 50 µl of saline solution and 100 µl of Chlorine Dioxide 0.25%, Sodium Hypochlorite 1% and Chlorhexidine 2% were used to make the following dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and test them with the following microorganisms, E. faecalis from root canal and *S. viridans*, anginosus group from the alveolus, in adittion, *Escherichia coli* ATCC and *Staphylococcus aureus* ATCC were included to observe bacterial growth after 18h of incubation.

Results: NaOCl inhibited the microorganisms in all the dilutions, the ClO_2 showed bacterial growth in dilution 1:128, CHX1:8, in *E. faecalis*, *S. viridans*, anginosus group, ClO_2:1:32, CHX:1:8, *Escherichia coli* ATCC ClO_2:1:32, CHX:1:128, *Staphylococcus aureus* ATCC ClO_2:1:64 and CHX:1:256.

Conclusion: NaOCl was the chemical agent that inhibited all the strains evaluated, followed by ClO_2 and Chlorhexidine 2% needed higher concentrations for the eradication of the analyzed strains.

Introduction

Most endodontic infections are treated by chemo-mechanical preparation, however, about 35-60% of the root canal walls remain intact [1,2]. Thus, failure of root canal treatment has been predominantly associated with an ineffective elimination of microorganisms in a planktonic state and biofilm, which are present in the entire root

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canal system [3-5]. Moreover, the development of apical periodontitis has also been observed in some apparently well endodontically treated teeth. About 5-15% [6] of retreatments are performed on teeth with previous apical periodontitis. Bacterial persistence following irrigation with NaOCl and Chlorhexidine has been shown to range from 30%-60% even when endodontic therapy has followed adequate standards [1]. Therefore, in most cases bacterial reduction can only be achieved after intracanal medication between appointments, calcium hydroxide has been the chemical substance most used for this purpose; however, other studies have shown inconsistent results regarding its effectiveness to significantly improve root canal disinfection [7,8]. Zhang, et al., [9] reported that the presence of microorganisms such as *M. timidum*, *S. intermedius* and *E. faecalis* predominate both in extraradicular biofilm and in periapical lesions. While *P. propionicus*, *A. adiacens*, *P. prevotii*, *C. gracilis*, and *P. aeruginosa* were found at significantly higher levels in extraradicular biofilm than in periapical lesions, *P. micra* and *A. rimae* were more abundant in periapical lesions. *Enterococcus faecalis*, is one of the most isolated bacteria from root canals with endodontic failure and periradicular infections [10,11], this microorganism can invade the root canal by penetrating dentinal tubules to a depth of 800 µm [12], which allows it to be safe from irrigating solutions and endodontic instruments. In addition, it owns great capacity to form biofilm [13] and capable of tolerate adverse environmental conditions, such as a dry climate, a high concentration of salts, or a high alkaline pH (such as that generated by calcium hydroxide) [14]. It has been shown that *E. faecalis* can survive below pH 11.5, but due to the buffering effect of dentin, alkalinity can only reach pH 10.3 after covering the root canal wall with calcium hydroxide [15]. This mechanism can be attribute to the ATP-bound proton/potassium antiport system that incorporates protons into cells to maintain intracellular pH in alkaline environments. Thus, cell membrane-bound proton transport systems are responsible for the resistance of *E. faecalis* to acids and alkalis. [16,17]. When these bacteria live in biofilm and are exposed to unfavorable conditions, they enter "starvation mode", capable of resisting up to 12 months inside the dentinal tubules, even after root canal filling [18]. Besides, it manages to recover quickly and potentiate their virulence mechanisms. Chlorine dioxide (ClO_2) is an inorganic compound containing 2 oxygen atoms and one chlorine atom, electronegative elements both. This chemical property of ClO_2 makes possible the release of oxygen when it decomposes during its action as an antimicrobial agent [19]. It is maintained within a wide pH range (4-10), and it is highly soluble in water. It owns the ability to oxidize other substances through a single electron transfer mechanism when dioxide is reduced to chlorite [20].

Chlorinated agents such as Cl_2 and Sodium Hypochlorite (NaOCl), used as disinfectants, react with organic matter to produce halogenated by-products such as trihalomethanes and haloacetic acids. A recent study showed that trihalomethane is highly carcinogenic, however, ClO_2 does not hydrolyze to form HCl, but remains as a true dissolved gas in solution and produces little or no trihalomethanes [21,22]. Ma, et al., [23] Demonstrated low ClO_2 toxicity through in vitro test, 50ppm ClO_2 did not cause eye irritation in rodents, neither exhibited abnormality nor mortality in 20ppm inhalation toxicity test of ClO_2, and UC-1 concentrations up to 40ppm were not toxic

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to mice for 90 days in the subchronic oral toxicity test. According to the characteristics described, ClO_2 could be used as root canal irrigant, therefore, the objective of this study was to compare the antimicrobial effectiveness of ClO_2, NaOCl and Chlorhexidine in microorganisms isolated from a persistent endodontic infection and in strains ATCC-type reference control, through in vitro antimicrobial sensitivity tests.

Materials and methods

An experimental-comparative in vitro study was carried out. For the antimicrobial sensitivity and minimum inhibitory concentration tests, reference control strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, and microorganisms isolated from an infection oral origin typified by the VITEK system were used. Sampling and Isolation of microorganisms from dental lesions. The microorganisms were isolated from a patient with endodontic failure (Figure 1 A and B), the apical third and the periapical granulation tissue were cultured in thioglycolate broth with meat and enriched with vitamin K (Figure 2), these samples were incubated for 48h at 37 °C, where the following microorganisms were recovered: *Enterococcus faecalis* from the apex and *Streptococcus viridans* anginosus group from the apical lesion (Figure 3).

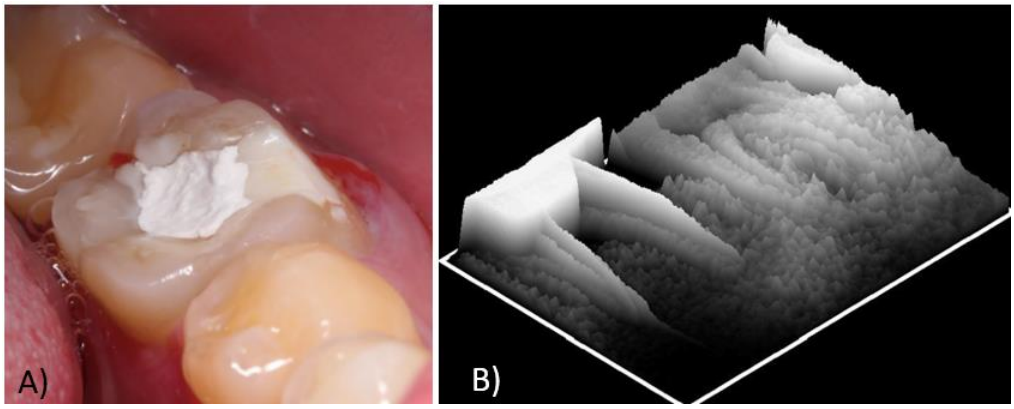

Figure 1: Lower right first molar, A) clinical view of vestibular abscess B) Pseudo 3D view, showing an extensive periapical lesion and overextension of filling material.

Figure 2: Apex and granulation tissue placed in thioglycolate broth with meat.

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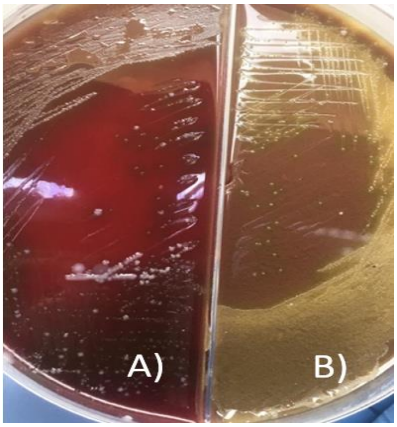

Figure 3: A) Enterococcus faecalis Brain Heart infusion Agar B) Streptococcus viridans anginosus group Chocolate Agar

Preparation of serial dilutions of antiseptics

The antiseptics selected for this study were: Chlorine Dioxide 0.25% (Irrigare, Biofilm Labs®), NaOCl 1% and Chlorhexidine 2% (Consepsis, Ultradent®), as negative control, 0.9% physiological saline solution was used.

50 µl of each evaluated solution was placed in 96-well polystyrene microplates; in each line, the concentrated solution was placed in well number 1 and subsequently serial dilutions were made 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, leaving well number 12 as growth control. (Figure 4A, B and C).

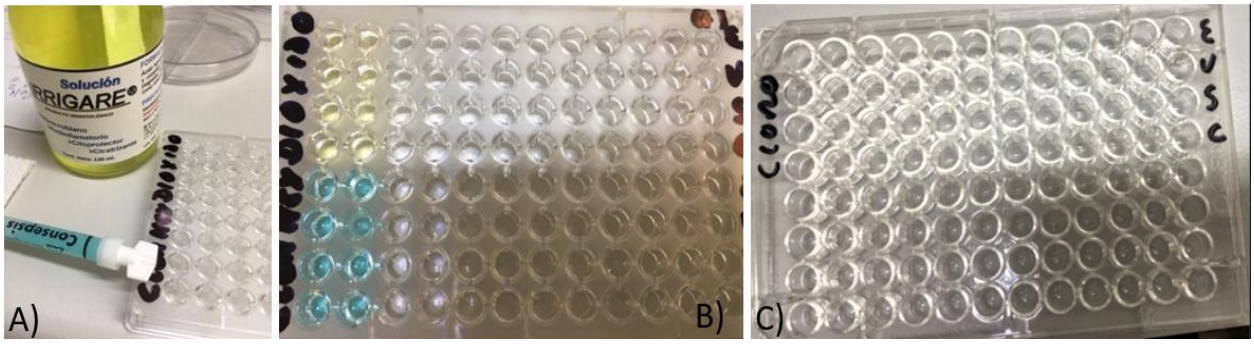

Figure 4: A) Tested Antiseptic Solutions, B) Serial Dilutions of ClO_2 and Clorhexidine C) Serial dilutions of NaOCl

Density of bacterial suspension

The density of each microorganism obtained from the culture was performed by means of DensiCHEK plus densitometer calibrated with Biomeriux Cat. standards, corresponding to the Clinical Laboratory Standard Institute (CLSI 2019).

In each well, 100 µl of each of the bacterial suspensions were added to the dilutions of the antiseptic solutions, at a concentration of 150,000,000 bacteria/mL (Scheme 1 and 2).

Culture method

To carry out the viable count, the microplates were incubated at 37 °C (Felisa®) for 15 to 30 min, subsequently, 1µL were taken from each well to be seeded in blood agar plates at 5%, (Figure 5 and 6), incubating each plate in reduced oxygen environment, at 37 °C for 18 hours.

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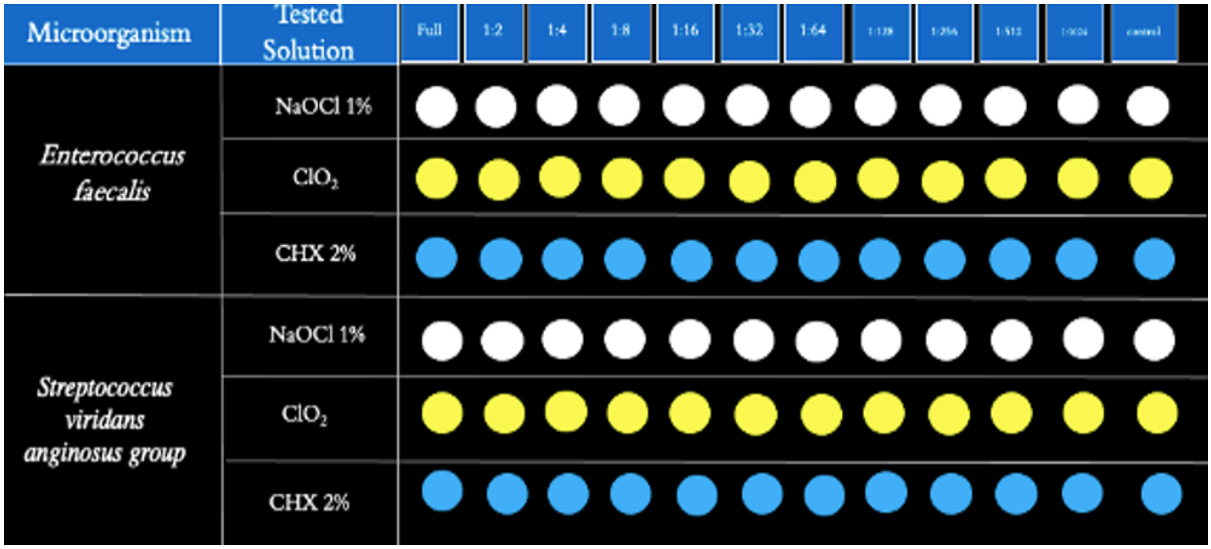
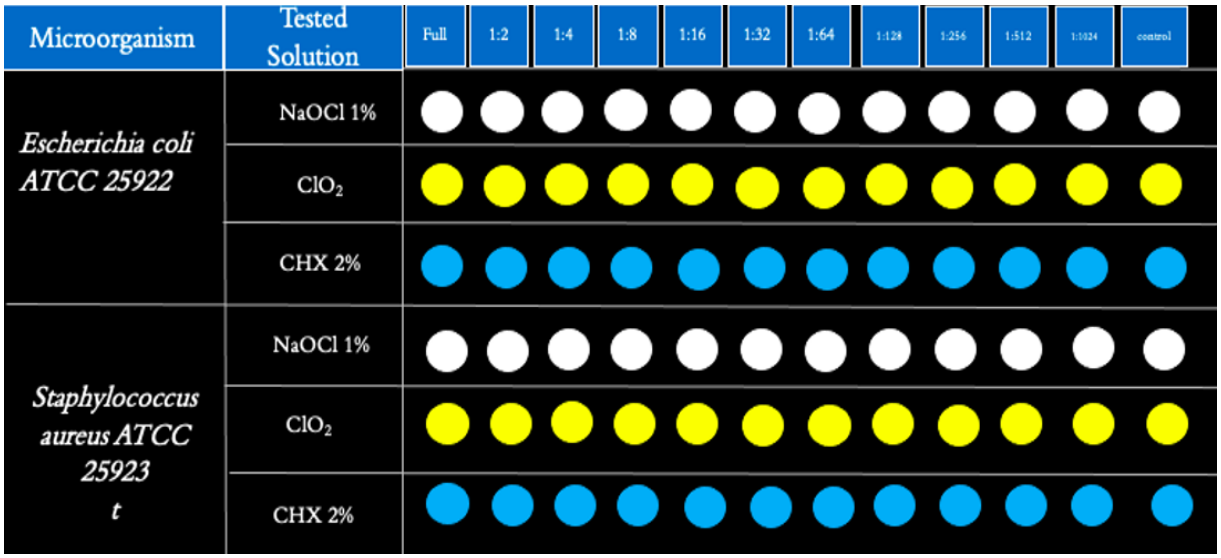


Scheme 1: Shows the comparisons between dilutions of each antiseptic solution and the bacterial suspension of the microorganisms isolated from the endodontic infection.



Scheme 2: Shows the comparisons between dilutions of each antiseptic solution and the bacterial suspension of ATCC reference strains.

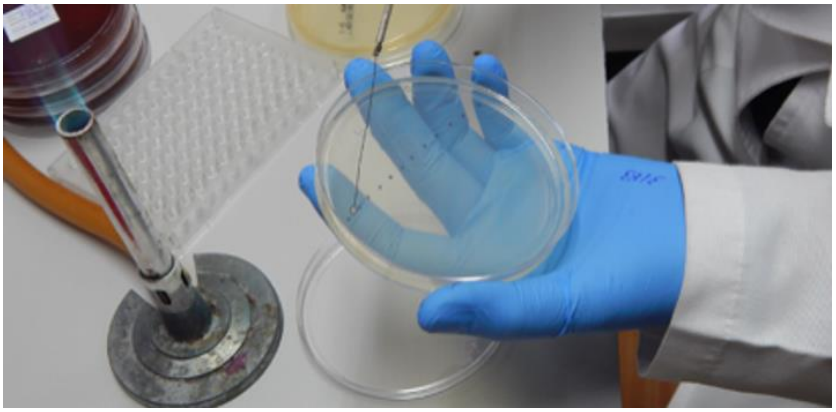


Figure 5: Bacteria Inoculation using Nichrome loop.

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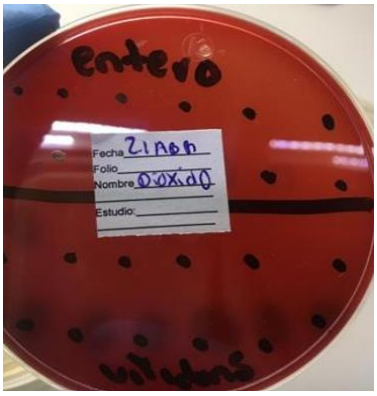

Figure 6: Blood Agar at 5%.

Subsequently, the isolated strains from oral cavity and ATCC were reseeded on Mueller Hinton Agar plates to quantify viable bacteria and growth inhibition.

Results

When evaluating the susceptibility through the Minimum Inhibitory Concentration, the 3 antimicrobial solutions presented susceptibility in their original concentrations both in the ATCC reference strains and strains of microorganisms isolated from biological samples of apex and alveolus, however, in the dilutions evaluated, ClO_2 showed inhibition of *E. faecalis* (Figure 7B-1) up to a dilution 1:128, in *S. Viridans* anginosus group (Figure 7B-2) and *Escherichia coli* ATCC 1:32 (Figure 8-1), for *Staphylococcus aureus* ATCC 1:64 (Image 8-2).

Regarding Chlorhexidine, it showed inhibition of E. faecalis (Figure 7C-1) and *S. Viridans* anginosus group (Figure 7C-2) at dilution 1:8, for ATCC strains in *Escherichia coli* it showed inhibition at 1:128 dilution and *Staphylococcus aureus* up to 1:256, being NaOCl (Figure 7A and 8) the solution that did not show bacterial growth in any of the evaluated dilutions (Table 1).

Discussion

In this study, NaOCl inhibited bacterial growth in all the evaluated dilutions, followed by ClO_2 0.25%, being chlorhexidine 2% the one that required the highest concentration to eliminate the microorganisms tested. The results agree with other studies where the effectiveness of NaOCl as antimicrobial agent has been demonstrated [24-31].

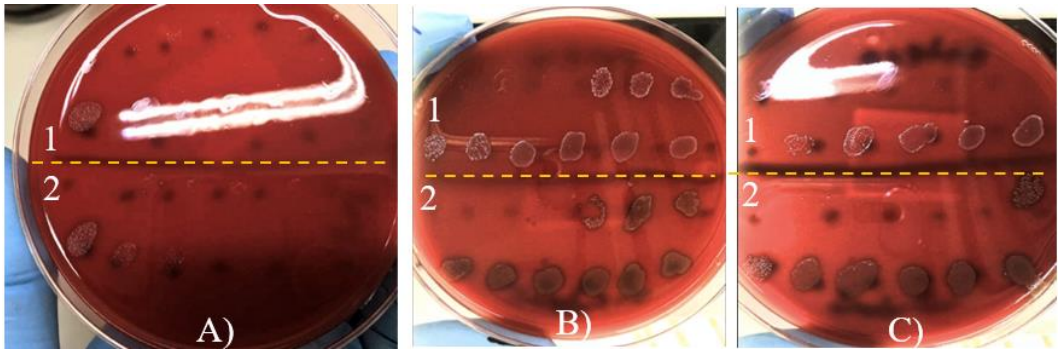

Figure 7: Shows the bacterial growth after the application of disinfectant solutions in blood agar plates, A) Sodium Hypochlorite (NaOCl), B) Chlorine Dioxide (ClO_2) and C) Chlorhexidine (CHX) in strains: 1) *E. faecalis* 2) *S. Viridans* anginosus group.

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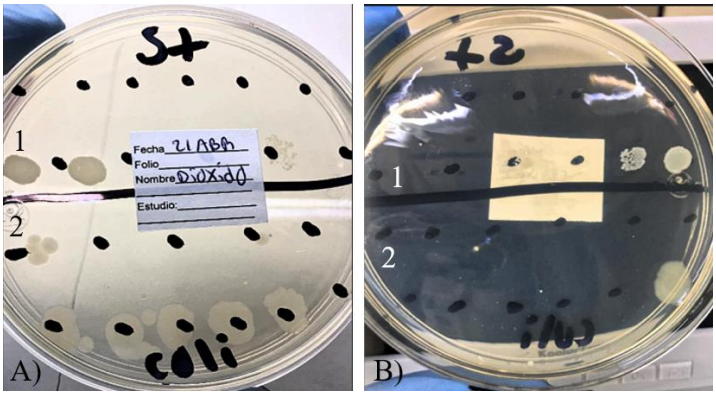

Figure 8: Shows the bacterial growth after the application of disinfectant solutions in ATCC strains: *Staphylococcus aureus* ATCC 25923 [1] and *Escherichia coli* ATCC 25922 [2]. A) Chlorine Dioxide and B) Sodium Hypochlorite.

The alkaline pH of NaOCl induces biosynthetic alterations in cell metabolism, protein denaturation, enzymatic inhibition, and destruction of phospholipids, [32] intervening in the integrity of the cytoplasmic membrane, which grants its bactericidal effect and tissue dissolution, for that reason, it is the chemical agent most used during endodontic therapy [33,34]. Nevertheless, being a non-selective disinfectant agent, NaOCl, can lead to cellular alterations such as chromosomal aberration, frequency of micronuclei, necrotic or apoptotic cells, and binucleated cells [35]. Even chlorhexidine, which has also been shown to be a good antimicrobial agent [36,37], can permanently stop cell migration and reduce the survival of fibroblasts, myoblasts, and osteoblasts [38]. For this reason, more biocompatible irrigating solutions have been sought; chlorine dioxide is an oxidizing agent with broad-spectrum bactericidal activity [39] with minimal or no side effects [40]. Lundstrom, et al., [41] noted that ClO_2 0.4%, exhibited little bactericidal activity against *P. nigrescens*, and was significantly less effective than NaOCl. In the other hand, Eddy, et al., [42] showed that NaOCl was more effective for bacterial inhibition than chlorine dioxide 13.8%, highlighting that chlorine dioxide can also eradicate E. faecalis in 30 min and in a higher concentration could eliminate it completely, later Ozkan, et al., [43] indicated that 10% and 13.8% chlorine dioxide and 5.25% NaOCl were effective in eliminating E. faecalis in 30 min, Somayaji, et al., [44] and Ersoy, et al., [45] also showed that NaOCl and ClO_2 caused almost the same bacterial inhibition (70%). Al-bayaty, et al., [46] found that ClO_2 gel had an effective antibacterial action against dental biofilm. Aberna, et al., [47] showed that ClO_2 effectively eradicated *E. faecalis*, improving its capacity after 1 min of exposure and in the absence of smear layer. On the other hand, a study made by Giardino, et al., [48] found that the effect of 5.25% NaOCl against Enterococcus faecalis is limited. According to Swimberghe, et al., [49] this microorganism is significantly less susceptible to NaOCl treatment when cultivated in a community of multiple species. To carry out the minimal inhibitory concentrations, *S. Viridans*, anginosus group and Enterococcus faecalis were isolated from a persistent apical periodontitis, *Escherichia coli* ATCC, and *Staphylococcus aureus* ATCC were included as well for evaluation. Francisco, et al., [50)] points out the importance of isolating

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microorganisms such as enterococcus directly from root canals since it can develop various virulence patterns such as the surface protein of enterococci (Esp) and the gelE gene which have increased potential to participate in colonization [51], while the multi-peptide resistance factor (MprF) protein confers resistance to antimicrobial peptides through electrostatic repulsion [52]. *E. faecalis* owns the capacity to use the dentin and periodontal ligament serum as a source of nutrition, ensuring its survival and allowing bacteria to adhere to and invade the dentinal tubules. In addition to synthesizing proteins, it secretes aggregation substance, sex pheromones, extracellular superoxide production, and the release of two important lytics: gelatinase and hyaluronidase [53]. Such stress proteins have been shown to be synthesized when exposed to acids and alkalis. Nevertheless, stress protein synthesis by *E. faecalis* appears not to be directly related to survival at extreme pH [54]. Not withstandig, Momenijavid, et al., [55] demonstrated that the components of Calcium Hydroxide [$Ca(OH)_2$], that is, Ca^{2+}, and alkaline pH-generating OH- hydroxyl groups, through a cooperative way strengthen the biofilm since the addition of Ca^{2+} causes cavities in the biofilm consequently an increase in cavities in the biofilm indicates the accumulation of large amounts of exopolysaccharides.

S. Viridans anginosus group allows the invasion of host cells, the evasion of immune activity, the spread and colonization of tissues [56]. It produces hyaluronidase (enzyme that destroys tissues and induces pus) and can favor the establishment of *C. albicans* in the oral cavity [57]. It has been shown that the mode of action of ClO_2 is through protein denaturation and involves selective covalent oxidative modification of cysteine, methionine, tyrosine, and tryptophan residues [58-60]. Several bacterial species generate lactic acid, acetic and other simple organic carboxylic acids. The acid medium triggers the decomposition of ClO_2 and the subsequent release of nascent oxygen, which is a particularly powerful oxidizing agent for anaerobic organisms because it is essentially a free radical that does not seek an electron; but two electrons [61]. Inhibits intracellular enzyme activities such as β-D-galactosidase. It also increases the permeability of the outer and cytoplasmic membranes, altering their integrity, leading to the exit of intracellular material and eventually can cause cell lysis [62]. It has been mentioned that unlike antibiotics, a special benefit of ClO_2 application is that it acts through mechanisms of action by which microorganisms cannot develop resistance in a classical way [63]. ClO_2 is a highly water-soluble gas, when it reaches 12 °C begins to separate from the water in its natural gaseous form. This ClO_2 gas owns the ability to diffuse and penetrate complex structures such as biofilm, dentin, periodontal tissue, or root canal disinfection, respectively. (Herczegh, et al., 2013) (Herczegh, et al., 2013). Besides, 0.25% chlorine dioxide has shown greater pharmacological potency in relation to 1% sodium hypochlorite and 2% chlorhexidine, since at much lower concentrations it achieves a great antimicrobial effect. This is a huge advantage that ClO_2 has over other antimicrobial molecules. On account of this and the results obtained in this study, solutions based on ClO_2 in aqueous medium could be used with great success in the root canal disinfection after the use of NaOCl, in order to potentiate the antimicrobial effect. Since NaOCl does not have the property of diffusion towards dentin and tissues, unlike

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ClO_2. Controlled clinical trials would be worthwhile to confirm the use of ClO_2 in a root canal irrigation protocol.

Conclusion

NaOCl 1% was the chemical agent that inhibited *Enterococcus faecalis* isolated from the apex and *Streptococcus viridans* anginosus group from the apical lesion, in the same way inhibited the growth of the reference strains: *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Chlorine dioxide 0.25% also showed a high effectiveness for the inhibition of these microorganisms, however, Chlorhexidine 2% needed higher concentrations for the eradication of these microorganisms.

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