UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara

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Root canal irrigation: physicochemical and biological properties of calcium hypochlorite and effects of the GentleWave system in endodontic treatment

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Root canal irrigation: physicochemical and biological properties of calcium hypochlorite and effects of the GentleWave system in endodontic treatment

Thesis presented to São Paulo State University (Unesp), School of Dentistry, Araraquara, to obtain the PhD degree in Dentistry, in the area of Endodontics.

Supervisor: Gisele Faria, PhD

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Eric Hernán Coaguila Llerena

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Thesis for obtaining the PhD degree in Dentistry

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CURRICULAR DATA

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I dedicate this work:

To God, in whose omnipresence, infinite benevolence and unquestionable will, I know that there is no impossible. God gave me nothing I wanted, he gave me everything I needed.

To my family, because it is through them that I learned to remain humble in victory and elegant in defeat. I know that the tireless pursuit of the realization of my dreams and the consolidation of the principles that guide my life are a consequence of what I learned at home. And, without a doubt, it is through them that I learned that I should never give up because God provides enough strength.

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"O que dá o verdadeiro sentido ao encontro é a busca, e é preciso andar muito para se alcançar o que está perto"
José Saramago*

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* Saramago J. Todos os nomes. Lisboa: Editorial Caminho; 1997.

Coaguila Llerena EH. Irrigação do canal radicular: propriedades físico-químicas e biológicas do hipoclorito de cálcio e efeitos do sistema GentleWave no tratamento endodôntico [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

RESUMO

Parte 1: Na publicação 1, o objetivo foi avaliar as propriedades físico-químicas e a penetrabilidade nos túbulos dentinários do hipoclorito de cálcio [$Ca(OCl)_2$] a 2,5% associado aos surfactantes cloreto de benzalcônio (BAK), cetrimida (CTR), tween 80 (T80) e triton X-100 (TR100), na concentração micelar crítica (CMC), em comparação ao hipoclorito de sódio (NaOCl) a 2,5%. Foram avaliados tensão superficial, pH, conteúdo de cloro livre (FAC), íons cálcio livres (Ca^{2+}) e a penetrabilidade nos túbulos dentinários. A adição de surfactantes reduziu a tensão superficial das soluções ($p<0,05$), e não alterou o pH e o FAC ($p>0,05$). Os surfactantes, principalmente BAK, aumentaram a disponibilidade de Ca^{2+} em $Ca(OCl)_2$ ($p<0,05$). $Ca(OCl)_2$ apresentou menor penetrabilidade nos túbulos dentinários que NaOCl ($p<0,05$) e a adição de surfactantes àquele não aumentou a sua penetrabilidade nos túbulos dentinários. Na publicação 2, o objetivo foi avaliar o mecanismo de citotoxicidade da solução de $Ca(OCl)_2$ em fibroblastos L929 e o seu efeito na biologia de osteoblastos-*like* (Saos-2), em comparação com NaOCl. Fibroblastos L929, expostos a $Ca(OCl)_2$ e NaOCl, foram avaliados quanto ao metabolismo celular, integridade dos lisossomos, tipo de morte, alterações no citoesqueleto e na ultraestrutura. O efeito das soluções sobre a atividade da fosfatase alcalina (ALP) foi determinada em Saos-2. O $Ca(OCl)_2$ promoveu maior viabilidade celular e menor porcentagem de apoptose e necrose do que o NaOCl ($p<0,05$). $Ca(OCl)_2$ e NaOCl diminuíram o metabolismo celular e a integridade dos lisossomos, induziram ruptura de microtúbulos e filamentos de actina, promoveram alterações do retículo endoplasmático rugoso e nas cristas mitocondriais, e não induziram a atividade da ALP. Concluiu-se que, embora a adição de surfactantes ao $Ca(OCl)_2$ não aumentou a sua penetrabilidade nos túbulos dentinários, eles promoveram menor tensão superficial, sem alterações nos valores de pH e FAC, além de maior disponibilidade de Ca^{2+} na solução. Adicionalmente, NaOCl e $Ca(OCl)_2$ apresentaram o mesmo mecanismo de citotoxicidade e não induziram atividade de ALP, porém, $Ca(OCl)_2$ foi menos citotóxico que NaOCl. **Parte 2:** Na publicação 3, o objetivo foi descrever, por meio de revisão de literatura, os efeitos do sistema GentleWave (GW) no tratamento endodôntico. GW mostrou resultados *in vitro* semelhantes ou melhores que a irrigação utrassônica passiva (PUI) sob diferentes aspectos. Clinicamente, embora GW tenha mostrado resultados promissores, ainda são necessários mais estudos. Na publicação 4, o objetivo foi avaliar a eficácia de remoção de biofilme multiespécie do GW e PUI. Molares inferiores humanos com configuração tipo II de Vertucci na raiz mesial foram inoculados com placa dental e incubados. As raízes mesiais foram instrumentadas até 20.06 (V-Taper) para o grupo GW, e até 35.04 (Vortex Blue) para o grupo PUI. Raspas de dentina foram obtidas pré e pós-tratamento para a análise por reação em cadeia da polimerase quantitativa (qPCR) e por sequenciamento do gene rRNA 16S (Next Generation Sequencing - NGS). Não houve diferença na remoção de biofilme entre GW e PUI ($p>0,05$). Pode-se concluir que mais pesquisas, principalmente clínicas, são necessárias para estabelecer se GW apresenta vantagens sobre outros métodos de irrigação.

Palavras chave: Biofilmes. Hipoclorito de cálcio. Hipoclorito de sódio. Permeabilidade da dentina. Sequenciamento de nucleotídeos em larga escala. Tensoativos. Teste de materiais.

Coaguila Llerena EH. Root canal irrigation: physicochemical and biological properties of calcium hypochlorite and effects of the GentleWave system in endodontic treatment [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

ABSTRACT

Part 1: In publication 1, the aim was to assess the physicochemical properties and the penetration into dentinal tubules of calcium hypochlorite [$Ca(OCl)_2$] at 2.5% associated with the surfactants benzalkonium chloride (BAK), cetrimide (CTR), tween 80 (T80) and triton X-100 (TR100), at critical micellar concentration (CMC), compared to sodium hypochlorite (NaOCl) at 2.5%. Surface tension, pH, free available chlorine (FAC), free calcium ions (Ca^{2+}) and penetration into dentinal tubules were evaluated. The addition of surfactants reduced the surface tension of the solutions ($p<0.05$), and did not change the pH and FAC ($p>0.05$). Surfactants, mainly BAK, increased the Ca^{2+} availability in $Ca(OCl)_2$ ($p<0.05$). $Ca(OCl)_2$ showed lower penetration into dentinal tubules than NaOCl ($p<0.05$) and the addition of surfactants to $Ca(OCl)_2$ did not increase its penetration into dentinal tubules. In publication 2, the aim was to assess the cytotoxicity mechanism of $Ca(OCl)_2$ solution on L929 fibroblasts and its effect on osteoblast-like (Saos-2) biology, compared to NaOCl. Cellular metabolism, lysosome integrity, type of cell death, changes in cytoskeleton and ultrastructure of L929 fibroblasts, exposed to $Ca(OCl)_2$ and NaOCl, were evaluated. The effect of the solutions on alkaline phosphatase (ALP) activity was determined in Saos-2. $Ca(OCl)_2$ promoted higher cell viability and lower percentage of apoptosis and necrosis than NaOCl ($p<0.05$). $Ca(OCl)_2$ and NaOCl decreased cellular metabolism and lysosome integrity, induced disruption of microtubules and actin filaments, promoted changes in the rough endoplasmic reticulum and mitochondrial cristae, and did not induce ALP activity. It was concluded that, although the addition of surfactants to $Ca(OCl)_2$ did not increase its penetration into dentinal tubules, they promoted lower surface tension, without changes in pH and FAC values, in addition to higher Ca^{2+} availability. Additionally, NaOCl and $Ca(OCl)_2$ showed the same mechanism of cytotoxicity, and they did not induce ALP activity; however, $Ca(OCl)_2$ was less cytotoxic than NaOCl. **Part 2:** In publication 3, the aim was to describe, through a literature review, the effects of the GentleWave system (GW) in endodontic treatment. GW showed similar or better *in vitro* results than passive ultrasonic irrigation (PUI) in different aspects. Clinically, although GW has shown promising results, further studies are still needed. In publication 4, the aim was to assess the effectiveness of multispecies biofilm removal of GW and PUI. Human mandibular molars with Vertucci type II configuration in the mesial roots were inoculated with dental plaque and incubated. Mesial roots were instrumented up to 20.06 (V-Taper) for GW group, and up to 35.04 (Vortex Blue) for PUI group. Dentin shavings were obtained pre- and post-treatment for analysis by quantitative real-time Polymerase Chain Reaction (qPCR) and 16S ribosomal RNA gene sequencing (Next Generation Sequencing - NGS). There was no difference in biofilm removal between GW and PUI ($p>0.05$). It can be concluded that more research, mainly clinical, is needed to establish whether GW has advantages over other irrigation methods.

Keywords: Biofilms. Calcium hypochlorite. Sodium hypochlorite. Dentin permeability. High-throughput nucleotide sequencing. Surface-active agents. Materials testing.

SUMMARY

1 INTRODUCTION

The control of infection is critical for the success of endodontic treatment[1], and the irrigation solution and its métodos de agitação used in chemo-mechanical preparation has an important role to achieve this goal[2].

Sodium hypochlorite (NaOCl) is the most commonly used irrigating solution due to its antimicrobial activity and organic dissolution capacity[3–5], being considered as "*gold standard*"[6]. However, NaOCl negatively alters the mechanical properties of dentine, such as microhardness, elastic modulus, resistance to flexion and fatigue[7], and can reduce the bond strength of root canal sealers[8] and some dentine adhesive materials[7,9]. Additionally, NaOCl does not provide adequate removal of the smear layer from the dentine surface[10], and when interacts with ethylenediaminetetraacetic acid (EDTA) causes deleterious effects to root canal dentine[11]. High concentrations of NaOCl are irritating when in contact with periapical tissues[12,13] and have a pronounced negative effect on the survival and differentiation of apical papilla stem cells, which can hinder pulp regeneration/revascularization[14]. The research for alternative irrigating solutions is focused on substances that have an antimicrobial effect, of organic dissolution, as well as biocompatibility.

Calcium hypochlorite [$Ca(OCl)_2$], a chlorine disinfectant[15], has been proposed as endodontic irrigant. It is available as granules powder and when prepared in aqueous solution, there is a release of hypochlorous acid and calcium hydroxide[4]: $Ca(OCl)_2 + 2\ H_2O \rightarrow 2HOCl + Ca(OH)_2$. Compared with sodium hypochlorite ($NaOCl + H_2O \rightarrow HOCl + NaOH$), there is a higher generation of hypochlorous acid[16]. The generated calcium hydroxide [$Ca(OH)_2$] could favour the antimicrobial activity of $Ca(OCl)_2$ [16]. In addition, calcium present in its composition, instead of sodium[17], could be associated to a possible osteogenic induction and subsequent mineralization in periapical tissues. The $Ca(OCl)_2$ solutions are highly alkaline (pH around 11-12), and higher surface tension than NaOCl[18]. $Ca(OCl)_2$ solution had a stable FAC up to 30 days, which is comparable to NaOCl stabilized with sodium hydroxide[18,19]. Studies have been performed assessing the $Ca(OCl)_2$ potential for tissue dissolution[4,20], efficacy against *Enterococcus faecalis*[20–22], effects on mechanical properties of root canal dentine[23,24], on composite resin microleakage[17], and cytotoxicity[25–28].

When used as irrigating solution in extracted teeth contaminated with *E. faecalis*, 2.5% $Ca(OCl)_2$ showed antimicrobial efficacy similar to 2.5% NaOCl[22], regardless of the use of passive ultrasonic irrigation (PUI)[21] or sonic activation using Vibringe system[29]. Another study showed that 2.5% $Ca(OCl)_2$ was more effective against *E. faecalis* than 2.5% NaOCl when used during chemo-mechanical preparation with Reciproc R40 file[30]. The 5% $Ca(OCl)_2$ has the same organic dissolution capacity as 5.25% NaOCl after 35 and 60 minutes of contact with the tissue[4]. This organic dissolution capacity gradually increases with time and with an increase in its concentration[31]. On the other hand, $Ca(OCl)_2$, does not have the ability to dissolve inorganic tissue and consequently does not remove the smear layer[16].

Regarding the effects on root canal dentine surface, $Ca(OCl)_2$ changes dentine roughness in a similar manner to NaOCl[23]. When used before an acetone-based adhesive system, $Ca(OCl)_2$ does not affect microleakage compared to NaOCl. However, the contact of $Ca(OCl)_2$ on dentine increases the amount of calcium and phosphorus ions, which can be beneficial for the mineralization process and for the formation of an amorphous calcium phosphate phase within the hybrid layer during bonding procedures[17]. Study showed that 6% NaOCl negatively affects the flexural strength, ultimate tensile strength and fracture resistance of dentine, while 6% $Ca(OCl)_2$ does not alter those properties[24].

Regarding $Ca(OCl)_2$ cytotoxicity, studies that used methyl-thiazole-tetrazolium - MTT[26–28] or trypan blue and scratch assay[25] do not show consensus. A study found no difference between $Ca(OCl)_2$ at a high concentration (5%) and NaOCl at a low concentration (0.5%) in L929 fibroblasts, that is, $Ca(OCl)_2$ had more cytocompatibility than NaOCl[27], which was corroborated by a study that used both solutions at the same concentration, 2.5%[26]. Another study that used 3T3 cells revealed that both $Ca(OCl)_2$ and NaOCl promoted similar cytotoxicity at 3h and 6h; however, at 24h, $Ca(OCl)_2$ promoted higher cell cytotoxicity[28]. On the other hand, no differences were founded between $Ca(OCl)_2$ and NaOCl in 3T3 fibroblasts[25].

In teeth with pulp necrosis, microorganisms can penetrate areas that are difficult to clean mechanically such as isthmus, ramifications, lateral or accessory canals, apical deltas and dentinal tubules[32]. It has been reported that the biofilm of *E. faecalis* and *Porphyromonas gingivalis* can invade dentinal tubules up to 500 µm deep[33]. Thus, the depth that irrigants penetrate into dentinal tubules is an important factor in endodontic treatment[34]. The penetration depth of NaOCl into dentinal tubules can be affected by concentration[33,34], contact time, temperature[34,35], agitation, use of gel form [36] and surface tension of the irrigant[35,37].

Inside root canal, a high surface tension can hinder the penetration of the irrigating solution into dentinal tubules, regions of isthmus and anatomical irregularities, reducing its antibacterial effectiveness[38]. It has been reported that a decreased surface tension can increase the penetration of the irrigating solution into inaccessible areas of the root canal system including dentinal tubules, improving the disinfection[35,37,38]. Substances that reduce the surface tension of irrigants, which are called surfactants, including cetrimide (CTR), benzalkonium chloride (BAK), triton X-100 (TR100) and tween 80 - T80 has been associated to irrigating solutions[36,39–42].

CTR is a cationic surfactant that significantly reduces the surface tension of NaOCl[38] and has antimicrobial activity[43]. Studies have shown that 0.2% CTR eradicated *E. faecalis* biofilm[43], improved the antimicrobial effect of 2% chlorhexidine against polymicrobial biofilm[42] and did not affect the 2.5% NaOCl activity against *E. faecalis* biofilm[44].

BAK is a cationic surfactant widely used in the medical field as a preservative for eye solutions[45] that has been mixed with NaOCl. The addition of 0.008% BAK reduced the contact angle and surface free energy, and had no effect on FAC, cytotoxicity and antimicrobial effectiveness of 2.4% NaOCl[46]. The mixture of 0.008% BAK with 6% NaOCl was more effective in eliminating *E. faecalis* from the root canal compared to 6% NaOCl alone[47].

T80 is a nonionic surfactant present in the composition of Biopure MTAD (Dentsply Sirona Endodontics, York, PA, USA)[48] and TR100, another nonionic surfactant, is present in Chlor-Xtra (Vista Dental Products, Racine, WI, USA)[49,50]; in both cases manufacturers do not disclose their concentrations. A previous study shows that ChlorXtra has lower surface tension than NaOCl without surfactant[38]. Another study revelead that the addition of 0.1% BAK, TR100 and T80 in 5% NaOCl reduced surface tension and did not change the pH and FAC in comparison to 5% NaOCl[39].

There is no consensus in the literature about the impact of surface tension on penetration of irrigating solutions into dentinal tubules[35–37]. Two studies showed that NaOCl solutions with surfactant had higher penetration into dentinal tubules[35,37], while another study showed that this addition had no effect on the penetration of NaOCl[36]. According to available literature, $Ca(OCl)_2$ had a higher surface tension than NaOCl at the same concentrations - 0.5%, 1%, 2.5% and 5.25%[18]. However, it is not known if $Ca(OCl)_2$ penetrates into dentinal tubules less than NaOCl. In addition, it is not known if the addition of surfactants would decrease the surface tension and increase the penetration of $Ca(OCl)_2$ into dentinal tubules, without changing the pH and FAC.

For the selection of irrigating solution for root canal treatment it must be considered not only its antimicrobial effectiveness and organic dissolution capacity, but also its possible cytotoxic effects since it may come in contact with periapical tissues[51], which may influence the prognosis of root canal treatment. This becomes even more critical in regenerative endodontics procedures, which are performed in immature teeth. This is because the irrigating solution also contacts the periapical tissues, which are essential for endodontic regeneration[52]. It is also necessary to consider the physicochemical properties of irrigating solutions such as pH, surface tension, FAC, as well as the ability to penetrate areas not touched by the instruments, which are fundamental factors for the disinfection of the root canal system.

As previously mentioned, the control of infection is necessary to promote the healing of periapical tissues affected by apical periodontitis[53]. However, disinfection may be challenging when bacteria are organized in multispecies matrix-enclosed communities, called biofilms, especially in teeth with complex anatomies. These bacterial structures can colonize the canal walls[54], ramifications and isthmuses[32].

To improve irrigation effectiveness, technologies such as ultrasonic activation have been used. Specifically, passive ultrasonic irrigation (PUI) improves the removal of debris, smear layer[55,56], and bacterial biofilm[57] from root canals. However, even with the use of ultrasonic activation associated with NaOCl, microorganisms, debris and even pulp tissue may remain in the isthmus, apical third[58], oval/flattened canals[59] and root canal curvatures[60]. Another aspect to be considered is that if a small apical enlargement is performed during conventional chemical-mechanical preparation, a large amount of bacterial biofilm and necrotic tissue may remain in the root canal[61].

The GentleWave (GW), a new system that combines multisonic and negative apical pressure (Sonendo Inc, Laguna Hills, CA, USA), was introduced on the US market in 2014, and represents a type of endodontic device developed for cleaning and disinfection of the root canal[62]. According to its manufacturer, GW can be used in situations that need only minimal instrumentation, instead of using conventional instrumentation[63].

GW uses high-speed fluid dynamics to deliver the irrigants into the root canal system without requiring the tip of the instrument to enter the root canals. The irrigant is delivered through a handpiece to the end of a nozzle placed in the sealed pulp[64], and the excess of irrigant is simultaneously removed from the chamber by the built-in vented suction through the handpiece into a waste canister inside the console[65]. GW creates a powerful shear force, which causes hydrodynamic cavitation in the form of a cavitation cloud. The implosion of thousands of microbubbles creates an acoustic field of broadband frequencies that travels through the procedure fluid into the entire root canal system[66].

Regarding microbial reduction, a study using real-time PCR and bacterial cultures in molars, revealed that GW promoted higher reduction of total microbial DNA than CUI[67]. In anterior teeth, GW promoted less bacterial reduction than PUI, as previously shown using next generation sequencing (NGS)[68].

Traditionally, culture methods have been used to assess the bacterial composition and decontamination of the root canal system [69]. This method allows a semi- or absolute quantification of culturable bacteria. However, a significant amount of microorganisms in the root canal space cannot be cultured under laboratory conditions. The development of The Human Genome Project [70] allowed the subsequent development of databases (i.e., SILVA) for use in conjunction with NGS technologies [71,72]. NGS is a fifth-generation laboratory tool of microbiological analysis for the study of endodontic infections. This method provides vast information about bacterial communities and their profiles[53,73]. To date, the decontamination efficacy of infected root canals irrigated with GW in molars has not been provided using a relevant infection model.

Studies have been conducted to assess other types of GW effects in endodontic treatment. The GW promoted a significantly fast rate of bovine muscle dissolution than conventional syringe irrigation (CSI), continuous ultrasonic irrigation (CUI) and negative-pressure irrigation[62]. The root canal treatment using GW was not associated with extrusion[65] since GW creates a negative pressure at the apical foramen, irrespective of canal instrumentation size[64]. GW removed more debris than CSI in minimally and conventionally instrumented canals[74]; as well as more debris than CUI, but no more than PUI in conventionally instrumented canals[75]. Additionally, GW showed a greater removal of calcium hydroxide paste in comparison to CSI and PUI, in the root canals submitted to conventional instrumentation[76]. A prospective multicenter clinical study showed that the treatment of teeth with large periapical lesions with GW resulted in a 97.7% success rate after 12-month re-evaluation[66]. The association of GW with 3% NaOCl without root canal instrumentation cleaned the organic matter (tissue remnants) and dentin debris even in irregular areas, especially in middle and apical thirds of premolars[77]. Regarding the penetration of NaOCl into dentinal tubules, the use of GW promoted greater penetration of 3% NaOCl than PUI and CUI[78].

Currently, GW costs approximately $80,000.00 per console, and $50.00 to $100.00 for a one-time use handpiece. However, several doubts have been raised in regard to GW: Is it worth investing in such high-cost equipment? Does it produce better results than conventional root canal treatment? What are the effects of GW on endodontic treatment? Has GW biofilm removal efficacy been evaluated using a relevant model?

2 PROPOSITION

The aims of the present thesis were:

Part 1:

-To determine the physicochemical properties and the penetration into dentinal tubules of 2.5% calcium hypochlorite [$Ca(OCl)_2$] mixed with the surfactants benzalkonium chloride (BAK), cetrimide (CTR), tween 80 (T80) and triton X-100 (TR100) at critical micelle concentration (CMC) (Publication 1).

-To determine the cytotoxicity mechanism of 2.5% $Ca(OCl)_2$ in L929 fibroblasts and its effect on the biology of human osteoblast-like - Saos-2 - (Publication 2).

Part 2:

-To describe the outcomes of the GentleWave system in endodontic treatment (Publication 3).

-To determine the multispecies biofilm removal efficacy of the GentleWave system and passive ultrasonic irrigation (Publication 4).

3 PUBLICATIONS

Part 1:

Publication 1. Physicochemical properties and penetration into dentinal tubules of calcium hypochlorite with surfactants.

Publication 2. Calcium hypochlorite cytotoxicity mechanism in fibroblasts and effect on osteoblast biology.

Part 2:

Publication 3. Outcomes of the GentleWave system on root canal treatment: a narrative review.

Publication 4. Multispecies biofilm removal by a multisonic irrigation system in mandibular molars

3.1 Publication 1*

PHYSICOCHEMICAL PROPERTIES AND PENETRATION INTO DENTINAL TUBULES OF CALCIUM HYPOCHLORITE WITH SURFACTANTS

Abstract

The aim was to assess the physicochemical properties and the penetration into dentinal tubules of calcium hypochlorite solution [$Ca(OCl)_2$], with or without surfactants. The surfactants benzalkonium chloride, cetrimide, Tween 80 and Triton X-100 were mixed at different concentrations with sodium hypochlorite solution (NaOCl), $Ca(OCl)_2$ and distilled water (control). Once the critical micellar concentration (CMC) of the surfactants in $Ca(OCl)_2$ and NaOCl was determined, pH, free chlorine, surface tension and free calcium ions were evaluated. The penetration into dentinal tubules of NaOCl and $Ca(OCl)_2$, with or without benzalkonium chloride and Triton X-100 [surfactants that promoted the lowest surface tension of $Ca(OCl)_2$], was assessed using human premolars stained with crystal violet. The statistical tests were one-way ANOVA and Tukey's post-test, Kruskal-Wallis and Dunn's post-test, two-way ANOVA and Bonferroni's post-test, and t-test; depending on the assay. The addition of surfactants reduced the surface tension of NaOCl and $Ca(OCl)_2$, and did not alter the pH or the free available chlorine of either solution. The addition of all surfactants increased the availability of free calcium ions in $Ca(OCl)_2$, especially benzalkonium chloride. $Ca(OCl)_2$ exhibited lower penetration into dentinal tubules than NaOCl, and the addition of surfactants did not improve the penetration of $Ca(OCl)_2$, but did increase the penetration of NaOCl. It can be concluded that the addition of surfactants to $Ca(OCl)_2$ did not increase the penetration into dentinal tubules, but it did promote lower surface tension, without changing the pH or free available chlorine values, and higher availability of free calcium ions in $Ca(OCl)_2$.

Keywords: Calcium hypochlorite; Dentin permeability; Endodontics; Sodium hypochlorite; Surface-active agents

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INTRODUCTION

Sodium hypochlorite solution (NaOCl) is the most commonly used endodontic irrigant in clinical practice (1). In water, NaOCl is dissociated in sodium hydroxide and hypochlorous acid. In this aqueous solution, hypochlorous acid partially dissociates into the hypochlorite anion. The free available chlorine is the sum of the hypochlorous acid and hypochlorite anion concentrations in the solution (2). Studies have demonstrated that higher values of free available chlorine are associated to a high antimicrobial activity (3), and organic tissue dissolution (2).

In recent years, calcium hypochlorite solution [$Ca(OCl)_2$] has been recommended as an endodontic irrigant, because it can dissolve organic tissue (4), and because of its antimicrobial effect (5). Additionally, $Ca(OCl)_2$ is less cytotoxic than NaOCl (6).

It has been suggested that high surface tension may hinder irrigant penetration into dentinal tubules, isthmuses and anatomical irregularities, resulting in reduced antibacterial effectiveness (7). For this reason, the penetration into dentinal tubules of NaOCl combined with surfactants [substances that reduce the surface tension] has been previously investigated, with favourable results found in some studies (8,9). Benzalkonium chloride, cetrimide, Tween 80 and Triton X-100 surfactants have already been combined with NaOCl (1,10,11).

The saturation point of a surfactant in any solution is called the critical micellar concentration (CMC); the concentration of a surfactant above this point does not decrease the surface tension significantly, and leads to the formation of micelles (10). It is important to determine the CMC of a surfactant in any solution, because the best wettability properties of the solution are obtained at the calculated concentration (10), and because the behaviour of the surfactant molecules is different in the presence of micelles (12). Although the CMC of benzalkonium chloride, Tween 80 and Triton X-100 in 2.4% NaOCl has been previously reported (10,11), there is no available literature on the CMC of these surfactants in 2.5% $Ca(OCl)_2$, or on the CMC of cetrimide in either 2.5% NaOCl or 2.5% $Ca(OCl)_2$.

In the study by Iglesias et al. (13), benzalkonium chloride and cetrimide were added to $Ca(OCl)_2$ at a concentration equal to that of the CMC of NaOCl. Both surfactants reduced the surface tension of 2.5% NaOCl and 2.5% $Ca(OCl)_2$, and did not alter their pH, free available chlorine, or pulp dissolution properties. Nevertheless, there is no available literature on the penetration into dentinal tubules of $Ca(OCl)_2$, with or without surfactants. Likewise, there is no available literature on Tween 80 and Triton X-100 in combination with $Ca(OCl)_2$.

Therefore, the present study aimed to assess the physicochemical properties as well as the penetration into the dentinal tubules of 2.5% $Ca(OCl)_2$, with or without surfactants at CMC, compared with 2.5% NaOCl. The null hypothesis was that there would be no difference among these solutions with or without the surfactants, regarding physicochemical properties of pH, free available chlorine, free calcium ions as well as penetration into the dentinal tubules.

METHODOLOGY (extended methodology in APPENDIX A)

Preparation of irrigation solutions, and determination of surface tension and CMC

The stock $Ca(OCl)_2$ and NaOCl were prepared at approximately 6% weight/volume (w/v). The $Ca(OCl)_2$ was prepared by diluting calcium hypochlorite powder (Êxodo Científica, Sumaré, SP, Brazil) in distilled water under constant agitation for 30 minutes, and then filtering the solution using filter paper to remove the sediment. The NaOCl was prepared by diluting a 10% NaOCl (AraQuímica, Araraquara, SP, Brazil) in distilled water. Then, the free available chlorine of both stock solutions was determined by using the iodine/sodium thiosulfate titration method, and the solutions were stored in a refrigerator for 20 days at most until use, at 4°C, protected from light. For the evaluation of the physicochemical properties, the solutions were taken from the refrigerator and kept in the room until the temperature of the solutions equalled the room temperature determined for each assay.

The 2.5% $Ca(OCl)_2$ and 2.5% NaOCl, with or without benzalkonium chloride, cetrimide, Tween 80 and Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), were prepared from stock solutions at different concentrations (Table 1).

Solution	Concentration of surfactant (%)
Water – benzalkonium chloride	0.001 – 2
Water – cetrimide	0.01 – 1
Water – Tween 80	0.000393 – 3
Water – Triton X-100	0.00312 – 3
2.5% NaOCl - benzalkonium chloride	0.001 – 1
2.5% NaOCl - cetrimide	0.0005 – 1
2.5% NaOCl - Tween 80	0.003 – 1
2.5% NaOCl - Triton X-100	0.00007 – 1
2.5% $Ca(OCl)_2$ - benzalkonium chloride	0.001 – 0.3
2.5% $Ca(OCl)_2$ - cetrimide	0.0001 – 0.2
2.5% $Ca(OCl)_2$ - Tween 80	0.003 – 1
2.5% $Ca(OCl)_2$ - Triton X-100	0.00007 – 1

Table 1. Concentration range of benzalkonium chloride, cetrimide, Tween 80 and Triton X-100, used to determine the critical micellar concentration (CMC) in water, in 2.5% sodium hypochlorite solution (NaOCl) and in 2.5% calcium hypochlorite solution [$Ca(OCl)_2$]

The surface tension of all the combinations (n = 3) was measured by using the pendant-drop method at 22°-24°C room temperature. Each solution was placed in a syringe coupled to an OCA-20 system (DataPhysics Instruments, Filderstadt, Germany), wherein it formed a drop digitally captured by a charge-coupled device. The surface tension was then calculated automatically based on the drop shape, using a SCA-20 software program (DataPhysics Instruments), as previously described (9). Next, the surface tension data were plotted in an Origin 8 software program (OriginLab, Northampton, MA, USA), and a linear regression of the curve was applied on both the “x” (surfactant concentration) and the “y” axes (surface tension). The intersection of the two lines (resulting from the “x” and “y” axes) allowed calculating the CMC, and determining the surface tension at CMC. The 2.5% NaOCl and the 2.5% $Ca(OCl)_2$, mixed with the surfactants at CMC, were used to assess pH, free available chlorine and free calcium ions.

Determination of pH

The freshly prepared 2.5% NaOCl and 2.5% $Ca(OCl)_2$, with and without surfactants at CMC (n = 3), were stirred. Then, the pH of each solution was measured using a pH-meter (DM-22, Digimed, São Paulo, SP, Brazil) at 22°C room temperature, according to the requirements of the European Pharmacopoeia.

Determination of free available chlorine

The free available chlorine was determined by using the iodine/sodium thiosulfate titration method (n = 3). A total of 10 mL of the diluted 2.5% NaOCl and 2.5% $Ca(OCl)_2$ (5g each in 100 mL of distilled water), with or without surfactants at CMC, was mixed with 30 mL of 5% potassium iodide (Êxodo Científica), and with 10 mL of 99.8% acetic acid (Neon Comercial, Suzano, SP, Brazil). Afterwards, a previously standardized 0.1 N sodium thiosulfate solution (Labsynth Produtos Para Laboratórios, Diadema, SP, Brazil) was dripped into the NaOCl and $Ca(OCl)_2$ using a standard 50 mL burette, until they became pale yellow. Immediately, a 0.5% starch solution (Êxodo Científica) was added, making the solution an intense blue colour. Subsequently, 0.1 N sodium thiosulfate (Labsynth) was dripped into the blue solution until it became transparent. The required sodium thiosulfate volume was recorded (10). The room temperature was maintained at 20°C (14). The data were expressed as a percentage (%) of w/v.

Determination of free calcium ions

The concentration of free calcium ions in the 2.5% $Ca(OCl)_2$ with or without surfactants at CMC (n = 3) was determined at 22°-24°C room temperature by potentiometry, using a calcium-selective electrode. The potentiometer (Bante Instruments, Sugar Land, TX, USA) allowed measuring free calcium ions conductivity (expressed as a mmol/L concentration) using a calibration curve (R^2=0.9996) taken from a standard calcium solution (0.1 M). Then, 10 µL to 40 µL of the solutions were added sequentially to 20 mL of distilled water, and an electrode was placed at each addition to obtain the measurements. The expected free calcium ions was calculated at each addition of the 20 mL of distilled water plus 10 µL to 40 µL of the experimental solutions. This theoretical calculation was performed considering that the formula for $Ca(OCl)_2$ has 2 moles of hypochlorite ion for 1 mole of calcium ion.

Penetration into dentinal tubules

This assay was performed by selecting the benzalkonium chloride and Triton X-100 surfactants, which promoted the lowest surface tension of both 2.5% NaOCl and 2.5% $Ca(OCl)_2$. The sample size was calculated using the G* Power 3.1.7 software program for Windows (Heinrich-Heine-Universität Düsseldorf, Germany). The calculation was based on an effect size = 0.44 (based on a pilot study), test power (β)

= 0.8, and α = 0.05, using the F-test family for one-way analysis, and showed that 72 specimens (n = 12) were required.

After approval of the study by the Ethics Committee of the School of Dentistry (CAAE: 09799019.4.0000.5416) (ATTACHMENT A), 75 freshly extracted human, permanent, single-rooted premolars donated by the tooth bank were scraped of any residual tissue tags, disinfected in 2.5% NaOCl for 5 min, and stored in 0.1% thymol at 4°C until use. The exclusion criteria comprised teeth with more than one root canal / apical foramen, oval-shaped canals, previous treatment, calcification, internal / external resorption, cracks, fractures on the root surface, > 5° Schneider angle, or canals that allowed the insertion of a file exceeding an ISO size 15 K-file into the apical foramen. To confirm the inclusion criteria, the teeth were examined using a stereomicroscope (Leica Microsystems, Wetzlar, Germany) and radiographed using a digital sensor (FONA CDR Elite, Schick by Sirona Dental, Long Island, NY, USA). Radiographic analysis was performed from mesiodistal and buccolingual projections, to select the teeth with similar dimensions, single, round-shaped canals, straight roots (< 5° Schneider angle), and single foramen (Vertucci's type I configuration). The radiographic images were analysed using an Image J software program (National Institutes of Health, Bethesda, MD, USA). The root canals were considered round-shaped when the buccolingual diameter equalled the mesiodistal diameter. After selection, the specimens were randomly allocated into six experimental groups (n = 12) using a computer algorithm (http://www.random.org) to ensure homogeneous distribution. The crowns were removed, and the root length was standardized at 16 mm. The root canals were instrumented using the ProDesign Logic system (Easy Equipamentos Odontólogicos, Belo Horizonte, MG, Brazil). At 15 mm working length, the 25.01, 25.03, 25.05 and 40.05 files were used at 350-600 rpm speed and 1-4 Ncm torque, depending on the file, using an electric motor (VDW Silver, VDW, Munich, Germany) (9). The root canals were irrigated with 2 mL of 2.5% NaOCl for 1 minute at each instrument change, followed by irrigation with 5 mL of 17% EDTA for 3 minutes, and 5 mL of distilled water. The canals were dried with paper cones equivalent to the last file. Next, the canals were filled with 1% crystal violet solution (Labsynth Produtos para Laboratórios, Diadema, SP, Brazil), and kept at 37°C and 95% relative humidity for 3 days. Then, they were irrigated with 20 mL of distilled water, and the apex was sealed with composite resin to create a closed system (1). The experimental groups were distributed as follows: 2.5% $Ca(OCl)_2$ +

benzalkonium chloride, 2.5% $Ca(OCl)_2$ + Triton X-100, 2.5% NaOCl + benzalkonium chloride, 2.5% NaOCl + Triton X-100, 2.5% $Ca(OCl)_2$ and 2.5% NaOCl, wherein the benzalkonium chloride and Triton X-100 surfactant groups used a previously determined CMC. Three teeth were irrigated with distilled water to serve as additional controls of the reaction. Next, the specimens were irrigated at 22-24°C room temperature with 5 mL of the irrigating solutions for 2 minutes (1) using a 5 mL syringe (Ultradent Products, South Jordan, UT, USA) coupled to a 27 G side-vented needle (Endo-Eze®, Ultradent Products), positioned 2 mm short of the working length. The root canals of all the groups were then irrigated with 5 mL of distilled water, and were sectioned transversely along their longitudinal axis at 3, 7 and 12 mm from the apex, using a low speed cutting machine (Isomet 1000, São Paulo, SP, Brazil), to obtain segments from the cervical, middle and apical segments. The cervical surface of each segment was polished using 1000-grit abrasive paper (3M ESPE, St. Paul, MN, USA), under constant irrigation with water. A stereomicroscope (LeicaM80, Leica Microsystems) and the Leica Application Suite EZ 3.0 software program (Leica Microsystems) were used to obtain the images. The penetration depth was measured in micrometres (µm) at 10 equidistant regions using the Image J program (National Institutes of Health, NIH) (9). A previously calibrated and blinded examiner performed the measurements twice, with a 2-week interval (intraclass correlation coefficient > 0.9).

Statistical analysis

The data were analysed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). An initial screening to assess data normality was performed by using the D'Agostino-Pearson test. The statistical tests used were one-way analysis of variance (ANOVA) and Tukey's post-test (surface tension, penetration into dentinal tubules – comparison among solutions), Kruskal-Wallis and Dunn's post-test (penetration into dentinal tubules – comparison among segments, showing no homogeneity of variance), two-way ANOVA and Bonferroni's post-test (free calcium ions), or the t-test (pH and free available chlorine), at a significance level of 5%.

RESULTS

Surface tension and critical micellar concentration

Figure 1 shows the surface tension of the water, and both the 2.5% NaOCl and 2.5% $Ca(OCl)_2$, combined with the surfactants at different concentrations. These data were used to calculate the CMC of the surfactants (Table 2). When the surfactants were combined with water, there was no difference between the CMC values of benzalkonium chloride and cetrimide ($p>0.05$), and both had a higher CMC values than Tween 80 and Triton X-100 ($p<0.05$). When combined with 2.5% NaOCl, all the surfactants showed different CMC values ($p<0.05$), but when combined with 2.5% $Ca(OCl)_2$, the benzalkonium chloride and Triton X-100 surfactants showed similar CMC values ($p>0.05$). The CMC values of benzalkonium chloride and cetrimide were similar in both 2.5% NaOCl and 2.5% $Ca(OCl)_2$ ($p>0.05$), whereas the CMC values of Tween 80 and Triton X-100 were different ($p<0.05$).

The surface tension of the water, of the 2.5% NaOCl and 2.5% $Ca(OCl)_2$, and of all these solutions combined with the surfactants at CMC, are shown in Table 2. Combined with water, Triton X-100 promoted a higher reduction in surface tension, followed by benzalkonium chloride and cetrimide, which were not different from each other ($p>0.05$), and next by Tween 80 ($p<0.05$). The 2.5% NaOCl showed higher surface tension than 2.5% $Ca(OCl)_2$ ($p<0.05$). The addition of the surfactants at CMC reduced the surface tension of 2.5% NaOCl and 2.5% $Ca(OCl)_2$ ($p<0.05$). It is important to note that benzalkonium chloride and Triton X-100 promoted the lowest surface tensions in both 2.5% NaOCl and 2.5% $Ca(OCl)_2$ ($p<0.05$). Mostly, 2.5% NaOCl with surfactants showed lower surface tension than 2.5% $Ca(OCl)_2$ with surfactants ($p<0.05$), except in the case of Triton X-100, which promoted a similar reduction in surface tension in both solutions ($p>0.05$).

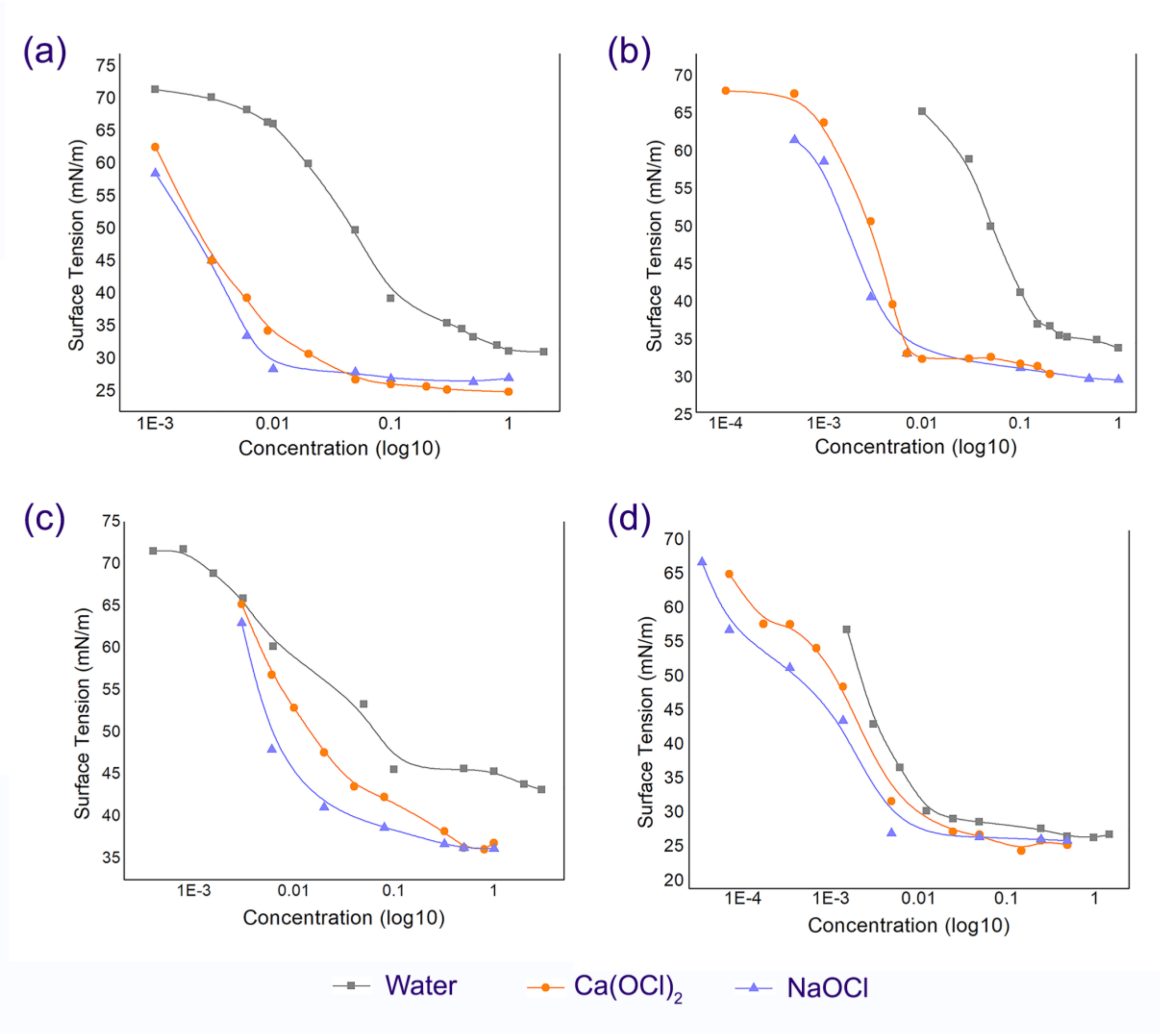

Figure 1. Effect of adding (a) benzalkonium chloride, (b) cetrimide, (c) Tween 80, and (d) Triton X-100 to water, 2.5% calcium hypochlorite solution [Ca(OCl)$_2$], and 2.5% sodium hypochlorite solution (NaOCl). The surface tension versus concentration (represented as log10 for better visualization) was used to determine the critical micellar concentration (CMC) of each surfactant in water, Ca(OCl)$_2$ and NaOCl.

Solution	Critical micellar concentration (%)	Surface tension (mN/m)
Water	–	72.81 (0.23) ^{a}
Water – benzalkonium chloride	0.1066 (0.0019%) ^{a}	35.87 (0.13) ^{b}
Water – cetrimide	0.1122 (0.0009%) ^{a}	36.22 (0.32) ^{b}
Water – Tween 80	0.0701 (0.0041%) ^{b}	45.89 (0.48) ^{c}
Water – Triton X-100	0.0249 (0.0004%) ^{c}	29.45 (0.33) ^{d}
2.5% NaOCl	–	69.66 (0.16) ^{aA}
2.5% NaOCl - benzalkonium chloride	0.0093 (0.0001%) ^{aA}	27.90 (0.21) ^{bA}
2.5% NaOCl - cetrimide	0.0065 (0.0005%) ^{bA}	30.74 (0.12) ^{cA}
2.5% NaOCl - Tween 80	0.0221 (0.0006%) ^{cA}	38.00 (0.41) ^{dA}
2.5% NaOCl - Triton X-100	0.0051 (0.0004%) ^{dA}	27.84 (0.39) ^{bA}
2.5% $Ca(OCl)_2$	–	68.30 (0.47) ^{aB}
2.5% $Ca(OCl)_2$ - benzalkonium chloride	0.0098 (0.0003%) ^{aA}	29.27 (0.24) ^{bB}
2.5% $Ca(OCl)_2$ - cetrimide	0.0066 (0.0001%) ^{bA}	32.42 (0.18) ^{cB}
2.5% $Ca(OCl)_2$ - Tween 80	0.0248 (0.0013%) ^{cB}	41.92 (0.83) ^{dB}
2.5% $Ca(OCl)_2$ - Triton X-100	0.0111 (0.0003%) ^{aB}	27.63 (0.07) ^{eA}

Table 2. Mean and standard deviation (in parentheses) of critical micellar concentration - CMC (in %) of benzalkonium chloride, cetrimide, Tween 80 and triton X-100in water, 2.5% sodium hypochlorite solution (NaOCl) and 2.5% calcium hypochlorite solution [$Ca(OCl)_2$], and the surface tension (in millinewton/meter) of the solutions with the surfactants at CMC.
Different lower case letters in the columns indicate significant differences among NaOCl, $Ca(OCl)_2$, water and associations with different surfactants ($p<0.05$). Different capital letters indicate significant differences between isolated 2.5% NaOCl and 2.5% $Ca(OCl)_2$, and between 2.5% NaOCl and 2.5% $Ca(OCl)_2$ associated with the same surfactant ($p<0.05$).

pH and free available chlorine

The addition of a surfactant did not change the pH of the 2.5% NaOCl or the 2.5% $Ca(OCl)_2$ ($p>0.05$). However, 2.5% NaOCl with and without a surfactant showed a higher pH, compared with 2.5% $Ca(OCl)_2$ with and without a surfactant ($p<0.05$). The addition of a surfactant did not alter the free available chlorine of either 2.5% NaOCl or 2.5% $Ca(OCl)_2$ ($p>0.05$), as shown in Table 3.

Solution	pH	Free available chlorine (% w/w)
2.5% NaOCl	12.33 (0.17) ^{aA}	2.54 (0.08) ^{aA}
2.5% NaOCl – benzalkonium chloride	12.25 (0.02) ^{aA}	2.52 (0.06) ^{aA}
2.5% NaOCl – cetrimide	12.36 (0.03) ^{aA}	2.50 (0.04) ^{aA}
2.5% NaOCl – Tween 80	12.42 (0.04) ^{aA}	2.56 (0.02) ^{aA}
2.5% NaOCl – Triton X-100	12.29 (0.05) ^{aA}	2.57 (0.04) ^{aA}
2.5% $Ca(OCl)_2$	11.76 (0.01) ^{bB}	2.61 (0.02) ^{aA}
2.5% $Ca(OCl)_2$ - benzalkonium chloride	11.76 (0.04) ^{bB}	2.55 (0.04) ^{aA}
2.5% $Ca(OCl)_2$ - cetrimide	11.84 (0.07) ^{bB}	2.62 (0.02) ^{aA}
2.5% $Ca(OCl)_2$ - Tween 80	11.79 (0.05) ^{bB}	2.57 (0.04) ^{aA}
2.5% $Ca(OCl)_2$ - Triton X-100	11.81 (0.04) ^{bB}	2.57 (0.04) ^{aA}

Table 3. Mean and standard deviation (in parenthesis) of physicochemical properties of pH and free available chlorine of 2.5% sodium hypochlorite solution (NaOCl) and 2.5% calcium hypochlorite solution [$Ca(OCl)_2$] with and without benzalkonium chloride, cetrimide, Tween 80 and Triton X-100 at critical micellar concentration (CMC).
Different lower case letters in columns indicate significant differences among NaOCl, $Ca(OCl)_2$ and associations with different surfactants ($p<0.05$). Different capital letters indicate significant differences between isolated 2.5% NaOCl and $Ca(OCl)_2$, and between 2.5% NaOCl and $Ca(OCl)_2$ associated with the same surfactant ($p<0.05$).

Free calcium ions

The use of a calcium-selective electrode allowed determining the availability of free calcium ions in the solution, and the effect of surfactant addition on this parameter. The results are shown in Figure 2. The addition of surfactants to 2.5% $Ca(OCl)_2$ increased the availability of free calcium ions, compared with 2.5% $Ca(OCl)_2$ without surfactants ($p<0.05$). In particular, the addition of benzalkonium chloride resulted in higher availability of free calcium ions in 2.5% $Ca(OCl)_2$, compared with the other surfactants ($p<0.05$).

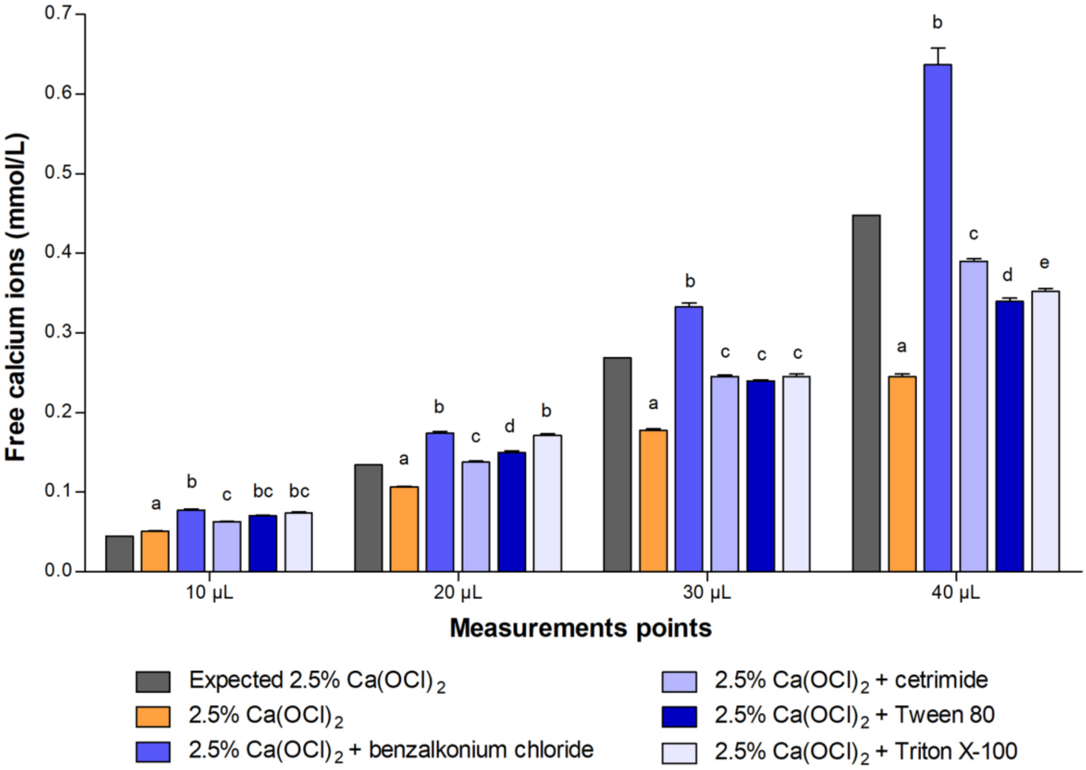

Figure 2. Mean and standard deviation of free calcium ions (mmol/L) of 2.5% calcium hypochlorite solution [$Ca(OCl)_2$] in association with benzalkonium chloride, cetrimide, Tween 80 and Triton X-100, at critical micellar concentration.
Different letters in each column indicate a significant difference among the solutions at each measurement point ($p<0.05$). The expected $Ca(OCl)_2$ values were not included in the statistical analysis.

Penetration into dentinal tubules

The 2.5% NaOCl + benzalkonium chloride and 2.5% NaOCl + Triton X-100 groups had the highest penetration depth in the cervical and middle segments, in comparison with the other groups ($p<0.05$). The penetration depth of 2.5% $Ca(OCl)_2$ was lower than that of 2.5% NaOCl in the cervical and middle segments ($p<0.05$). In these segments, there were no differences among 2.5% $Ca(OCl)_2$, with or without surfactants ($p>0.05$). In the apical segment, there were no significant differences among any of the groups ($p>0.05$). Comparison of the segments revealed that the penetration depth in the cervical and middle segments was no different for 2.5% NaOCl with or without surfactants ($p>0.05$), but it was different for these groups in the apical segment ($p<0.05$). The 2.5% $Ca(OCl)_2$ group had a higher penetration depth in the cervical segment ($p<0.05$), whereas there were no differences in the middle and apical segments ($p>0.05$). The 2.5% $Ca(OCl)_2$ + benzalkonium chloride group showed no differences between the cervical and middle segments, or between the middle and apical segments ($p>0.05$). The penetration depth of 2.5% $Ca(OCl)_2$ +

Triton X-100 group was not different between the cervical and the middle segments, but was lower in the apical versus cervical or middle segments ($p>0.05$) (Figure 3 and Figure 4).

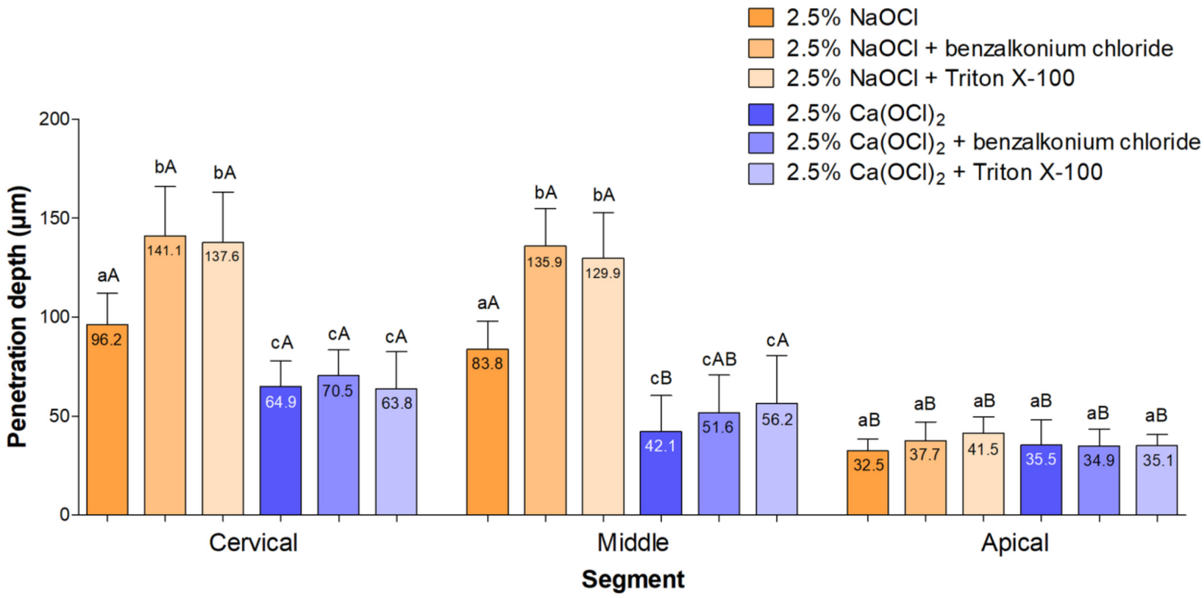

Figure 3. Mean and standard deviation in micrometres (µm) of penetration depth into dentinal tubules of 2.5% sodium hypochlorite solution (NaOCl) and 2.5% calcium hypochlorite solution [$Ca(OCl)_2$] combined with benzalkonium chloride or Triton X-100. The mean value is shown in each column. Different lowercase letters in columns of each segment indicate a significant difference among the solutions. Different uppercase letters in columns indicate a significant difference for each solution.

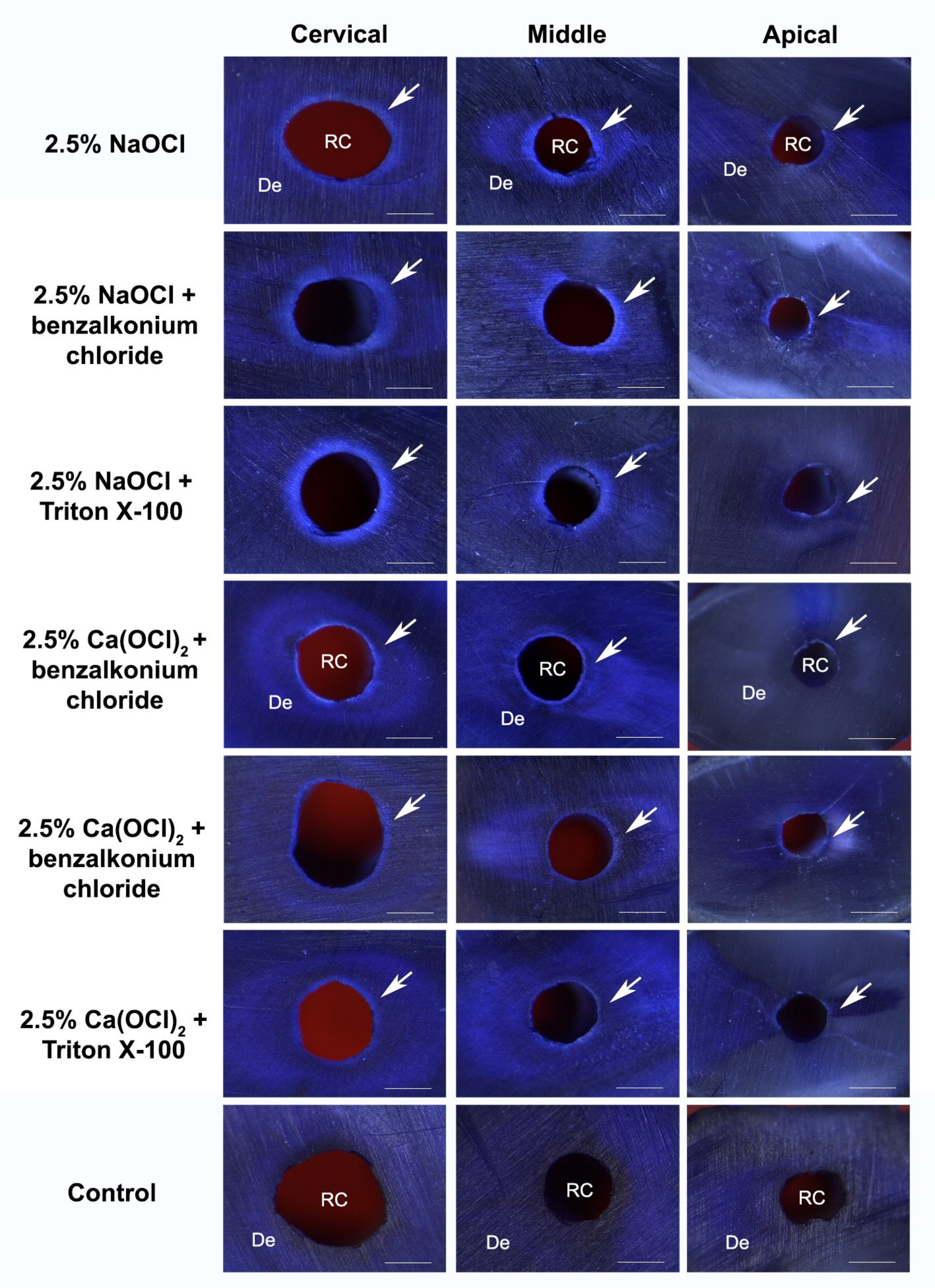

Figure 4. Representative images of penetration depth into dentinal tubules of 2.5% sodium hypochlorite solution (NaOCl) and 2.5% calcium hypochlorite solution [$Ca(OCl)_2$] combined with benzalkonium chloride or Triton X-100 in cervical, middle and apical segments. The bleached crystal violet represents the penetration depth of irrigants into the dentine (arrow) (bar = 500 µm).
De, dentine; RC, root canal.

DISCUSSION

The CMC of the surfactants in 2.5% NaOCl and 2.5% $Ca(OCl)_2$, and the surface tension of 2.5% NaOCl and 2.5% $Ca(OCl)_2$ with surfactants at CMC was determined in the first part of the study. Then, the physicochemical properties of pH, free available chlorine, and free calcium ions of 2.5% NaOCl and 2.5% $Ca(OCl)_2$ with or without surfactants at CMC were evaluated. Based on these initial findings, two surfactants (benzalkonium chloride and Triton X-100) were selected to assess penetration of the irrigants into the dentinal tubules, because they provided the lowest surface tension of 2.5% $Ca(OCl)_2$ and 2.5% NaOCl. The null hypothesis was partially rejected, because there were some differences in the parameters for the solutions studied.

It has been reported that $Ca(OCl)_2$ forms a white precipitate at room temperature (25°C), even after filtering (13,15), and that this may affect the equilibrium concentration of the solution (13). To minimize the formation of this precipitate, $Ca(OCl)_2$ was stored at 4°C until use (15). NaOCl was also stored at 4°C until use, to counteract the instability of the solution (15).

The mechanism of the irrigating solution combined with the surfactant causes the surfactant to be adsorbed on the liquid/air interface of the solution, leading to a decrease in the surface tension of the solution (12,16). The adsorbed surface remains in equilibrium with the surfactant in the solution, as the concentration of the surfactant is gradually increased to a saturation point called CMC (16). The CMC of benzalkonium chloride and of cetrimide were similar in 2.5% NaOCl and 2.5% $Ca(OCl)_2$, whereas the CMC of Tween 80 and Triton X-100 were higher in 2.5% $Ca(OCl)_2$. The CMC of benzalkonium chloride in 2.5% NaOCl (0.0093%) was relatively close to the previously reported 0.008% in 2.4% NaOCl (10). On the other hand, the CMC of Tween 80 (0.0221%) and Triton X-100 (0.0051%) observed in our study using 2.5% NaOCl was different from the CMC for Tween 80 (0.01%) and Triton X-100 (0.00035%) previously reported by Bukiet et al.(11) using a 2.4% NaOCl. The differences could be attributed to the high temperature sensitivity of Tween 80 and Triton X-100, both ethoxylated non-ionic surfactants (12). In the present study, measurements were performed at 22-24°C room temperature (9), while the study by Bukiet et al.(11) was performed at 37°C. Regarding the CMC of cetrimide in 2.5% NaOCl (0.0065%), there is no available literature concerning this association.

Among the physicochemical properties of chlorinated solutions in endodontics, the pH factor may affect the free available chlorine (17), because it is directly related to the dissociation of hypochlorous acid (2). In the present study, 2.5% NaOCl and 2.5% $Ca(OCl)_2$ were alkaline, as corroborated by previously reported literature (9,13,15), and the addition of surfactants did not affect the pH, which also agrees with previous studies (8,13). The surfactant did not influence the pH of either the 2.5% NaOCl or the 2.5% $Ca(OCl)_2$, because of its low concentration (13). However, NaOCl with and without surfactants had a higher pH than the equivalent $Ca(OCl)_2$. These results diverge from those of the study by Leonardo et al. (15), who observed that 2.5% NaOCl and 2.5% $Ca(OCl)_2$ showed similar pH at 25°C. The differences could be attributed to the free available chlorine values, since Leonardo et al.(15) reported that 2.5% $Ca(OCl)_2$ had higher free available chlorine values than 2.5% NaOCl, whereas the free available chlorine values found in the present study were similar for both solutions, and very close to what was expected. It has been reported that there is a correlation between low pH and low free available chlorine (18); this being the case, the correlation may have influenced the comparison.

The addition of surfactants did not alter the free available chlorine of either 2.5% NaOCl or 2.5% $Ca(OCl)_2$, in agreement with previous studies (10,13). Moreover, 2.5% NaOCl and 2.5% $Ca(OCl)_2$ showed similar free available chlorine, as expected, since the solutions were prepared from stock solutions with known free available chlorine values. However, it is important to note that the stock $Ca(OCl)_2$ should have had a 6% concentration, but it was actually lower (data not shown); the resulting concentration corroborated a study showing that 5% and 10% $Ca(OCl)_2$ both had lower free available chlorine than expected (4). It is important to emphasize that one of the aims of this laboratory study was to evaluate the influence of the addition of surfactants in NaOCl and $Ca(OCl)_2$ on pH and free available chlorine; however, a clinical extrapolation, is not possible since it has been shown that dentine debris and pulp tissue can affect those properties (19,20). This may be considered a limitation of the present study.

The addition of surfactants, especially benzalkonium chloride and Triton X-100, significantly decreased the surface tension of 2.5% NaOCl and 2.5% $Ca(OCl)_2$, as observed in a previous study using 2.4% NaOCl combined with benzalkonium chloride (10). Yet another prior study reported a reduced surface tension for 2.5% $Ca(OCl)_2$ combined with benzalkonium chloride and cetrimide, although the authors used surfactant concentrations different from that corresponding to the CMC (13). In the present study, benzalkonium chloride, cetrimide, Tween 80 and Triton X-100 surfactants were added to 2.5% $Ca(OCl)_2$ at CMC. The combination of surfactants could be an alternative to be considered. However, a study revealed that the combination of benzalkonium chloride, Triton X-100, ethyl formate and polyethylene glycol in 5.25% NaOCl might not be appropriate, since only benzalkonium chloride promoted the necessary reduction in surface tension (21).

The 2.5% $Ca(OCl)_2$ solution showed lower surface tension than 2.5% NaOCl, which disagrees with studies that showed opposite results (13,15). The differences could be attributed to the methodology used, since the two studies used the ring method, whereas the present study used the pendant drop method. Although those studies were performed at 25°C room temperature and were in agreement regarding the surface tension of 2.5% $Ca(OCl)_2$, they disagreed from each other regarding the surface tension of 2.5% NaOCl, namely: 64.68 mN/m (15) and 46.30 mN/m (13).

The measurement of free calcium ions is an important parameter to be considered, since it is involved in the mineralization repair processes by promoting osteoblastic and odontoblastic differentiation and mineralization of undifferentiated mesenchymal cells (22,23). All the surfactants increased the availability of free calcium ions in 2.5% $Ca(OCl)_2$, but more pronouncedly for benzalkonium chloride. This can be explained by the cationic nature of the positively charged polar head of benzalkonium chloride (12), which may have induced repulsive interaction with the divalent calcium ion, thus hindering its binding to this ion. This effect was less pronounced in the case of Tween 80 and Triton X-100, which are non-ionic surfactants (12), and may interact with calcium ion in solution through ion-dipole interactions. The interaction and binding of calcium ion by hydroxyl groups has been described previously (24). In the case of cetrimide, its lower CMC compared with benzalkonium chloride may have influenced its behaviour in solution; however, more research is needed to substantiate this.

The crystal violet staining to assess penetration of the irrigants into dentine has been used previously (1,9,25,26). Both NaOCl and $Ca(OCl)_2$ are chlorinated solutions containing the hypochlorite ion, a powerful oxidizing agent that bleaches the colour of crystal violet, revealing the normal light colour of dentine (25). In other words, since NaOCl and $Ca(OCl)_2$ have to penetrate into the dentine to discolour it, the resultant bleached area can be correlated with penetration depth (1). The crystal violet staining has limitations such as the indirect assessment of the penetration through the bleached area, which is not necessarily correlated to antimicrobial activity, and the variable amount and diameter of dentinal tubules of each specimen, which may influenced the results. The contact time of the solutions in dentine was 2 min, as previously reported (1,9).

The 2.5% $Ca(OCl)_2$ had a lower penetration depth into dentinal tubules than 2.5% NaOCl in the cervical and middle segments. The addition of benzalkonium chloride and Triton X-100 promoted a higher penetration of 2.5% NaOCl into dentinal tubules, in agreement with previous studies that used NaOCl with surfactants in those segments (8,9). Interestingly, the addition of surfactants did not improve the penetration of 2.5% $Ca(OCl)_2$ into dentine. The low penetration of both $Ca(OCl)_2$ and NaOCl with or without surfactant in the apical segment could have been influenced by the sclerotic dentine inherent in that segment (27).

To the best to our knowledge, there is no study that evaluated the penetration of 2.5% $Ca(OCl)_2$ into dentine, hence precluding a proper comparison. The low penetration into dentinal tubules of $Ca(OCl)_2$, with or without surfactants, could be attributed to the formation of calcium hydroxide precipitate when $Ca(OCl)_2$ is at 25°C (4,13), hence obliterating the opening of dentinal tubules. Although the penetration depth of $Ca(OCl)_2$ into dentinal tubules was lower than that of NaOCl, further research is needed to address the antimicrobial activity of $Ca(OCl)_2$ along the extension of the dentinal tubules using confocal laser scanning microscopy. On the other hand, considering that the addition of surfactants led to a higher availability of free calcium ions, further research is also required to assess whether $Ca(OCl)_2$ combined with surfactants has an effect on osteogenic and odontogenic differentiation of mesenchymal stem cells, rendering an outcome that could favour a repair process in endodontics.

CONCLUSIONS

All the surfactants researched reduced the surface tension of NaOCl and $Ca(OCl)_2$, without changing the pH or free available chlorine values, and allowed higher availability of free calcium ions in the $Ca(OCl)_2$, especially benzalkonium chloride. $Ca(OCl)_2$ had lower penetration into the dentinal tubules than NaOCl, while the benzalkonium chloride and Triton X-100 surfactants did not affect the penetration of $Ca(OCl)_2$, but did increase the penetration of NaOCl.

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3.2 Publication 2*

CYTOTOXICITY MECHANISM OF CALCIUM HYPOCHLORITE IN FIBROBLASTS AND EFFECT ON OSTEOBLAST BIOLOGY

Abstract

Aim To determine the cytotoxicity mechanism of 2.5% calcium hypochlorite [$Ca(OCl)_2$] in L929 fibroblasts, and the effect of this solution on the biology of human osteoblast-like cells (Saos-2), compared to that of 2.5% sodium hypochlorite (NaOCl).

Methodology L929 fibroblasts were exposed to $Ca(OCl)_2$ and NaOCl at different dilutions for 10 min. Cell metabolism was assessed by methyl-thiazole-tetrazolium (MTT); lysosome integrity, by neutral red (NR) assay; type of cell death, by flow cytometry (apoptosis/necrosis); cytoskeleton, by actin and α-tubulin fluorescence; and cell ultrastructure, by transmission electron microscopy (TEM). The alkaline phosphatase (ALP) activity induced by $Ca(OCl)_2$ was determined in Saos-2 by thymolphthalein release. The data were analysed by two-way ANOVA and Bonferroni's post-test ($\alpha=0.05$).

Results $Ca(OCl)_2$ promoted higher cell viability, and a lower percentage of apoptosis and necrosis than NaOCl ($P < 0.05$). $Ca(OCl)_2$ and NaOCl decreased cell metabolism and lysosome integrity, induced the breakdown of microtubules and actin filaments, promoted alterations of rough endoplasmic reticulum and disruption of mitochondrial cristae, and did not induce ALP activity.

Conclusions Although $Ca(OCl)_2$ and NaOCl promoted the same cytotoxicity mechanism and did not stimulate ALP activity, $Ca(OCl)_2$ was less cytotoxic than NaOCl.

Key words: calcium hypochlorite, cell culture techniques, materials testing, root canal treatment, sodium hypochlorite

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Introduction

Traditionally, sodium hypochlorite (NaOCl) has been highly recommended for the irrigation of the root canal system, owing to its adequate organic dissolution and antimicrobial activity (del Carpio-Perochena et al., 2015), factors that have led to its being considered the “gold standard”. However, if extruded, it can be highly irritating to the periapical tissues, depending on the concentration, volume and pressure (Guivarc’h et al., 2017); high concentrations of NaOCl, e.g., 5.25% or 6%, have promoted high cytotoxic effects (Martin et al., 2014; Coaguila-Llerena et al., 2020). Currently, there is no available ideal endodontic irrigant, and alternative solutions are under research.

Calcium hypochlorite [$Ca(OCl)_2$] is a chlorine disinfectant (Tyan et al., 2018) that has been proposed as an endodontic irrigant. It is available in the form of powder granules that are prepared with an aqueous solution to trigger the release of hypochlorous acid and calcium hydroxide (Dutta & Saunders, 2012), which may be associated to its high pH – about 11-12 (Leonardo et al., 2016). In previous studies, the $Ca(OCl)_2$ solution provided stable, free available chlorine for up to 30 days, which is comparable to NaOCl stabilised with sodium hydroxide (Iqbal et al., 2016; Leonardo et al., 2016). As an endodontic irrigant, $Ca(OCl)_2$ promoted antibacterial activity against *Enterococcus faecalis* (De Almeida et al., 2014; Dal Bello et al., 2019, De Paula et al., 2019), and organic dissolution capacity (Dutta & Saunders 2012, De Paula et al., 2019). $Ca(OCl)_2$ also provides antimicrobial effects when used as an intracanal medication for regenerative endodontics procedures, or when added to endodontic sealers (Silva et al., 2021; Alfadda et al., 2021). Regarding the cytotoxic effects of $Ca(OCl)_2$, the literature shows divergent data, i.e., lower, similar, or higher cytotoxicity than NaOCl (Sedigh-Shams et al., 2016; Blattes et al., 2017; Coaguila-Llerena et al., 2019; Yilmaz et al., 2020). Currently, there is no study that has evaluated the cytotoxicity mechanism of $Ca(OCl)_2$.

A foremost, ongoing concern has been to characterise the cell damage induced by an endodontic irrigant, because it can come into contact with the host’s periapical tissues, and may alter physiological processes and enzymatic reactions that can cause an inflammatory response (Chang et al., 2001; Bapat et al., 2021). This is more critical in regenerative endodontics or apexification procedures performed on teeth with an open apex, because there is a high risk of extrusion of the endodontic irrigant beyond the periapical tissues (Scott et al., 2018). An endodontic irrigant should not interfere with the cell mineralisation capacity, which is important to achieve a tight seal of the apical region after obturation (Yasuda et al., 2010). This study aimed to determine the cytotoxicity mechanism of 2.5% $Ca(OCl)_2$ in L929 fibroblasts, and the effect of this solution on the biology of human osteoblast-like cells (Saos-2), compared with 2.5% NaOCl. The null hypothesis was that there would be no difference between the solutions.

Materials and methods (extended methodology in APPENDIX B)

This study was reported in accordance with the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021). The PRILE 2021 flowchart is presented in Figure 1.

PRILE 2021 Flowchart

RATIONALE/JUSTIFICATION

Currently, there is no study that has evaluated the cytotoxicity mechanism of calcium hypochlorite [$Ca(OCl)_2$] and its effect on the biology of human osteoblast.

AIM/HYPOTHESIS

To determine the cytotoxicity mechanism of 2.5% $Ca(OCl)_2$ in L929 fibroblasts, and the effect of this solution on the biology of human osteoblast-like cells (Saos-2), compared with 2.5% sodium hypochlorite (NaOCl). The null hypothesis was that there would be no difference between the solutions.

ETHICAL APPROVAL

Not applicable

SAMPLES

Serial dilutions of $Ca(OCl)_2$ and NaOCl (for assessment of cell metabolism, lysosome integrity, type of cell death, cytoskeleton, and cell ultrastructure).

$Ca(OCl)_2$ and NaOCl at 0.00002% [for assessment of alkaline phosphatase (ALP) activity].

EXPERIMENTAL AND CONTROL GROUPS, INCLUDE INDEPENDENT VARIABLES

NaOCl Group – Applied to L929 fibroblasts or Saos-2, in triplicate

$Ca(OCl)_2$ Group – Applied to L929 fibroblasts or Saos-2, in triplicate

Control Group (culture medium/saline) – Applied to L929 fibroblasts or Saos-2, in triplicate

OUTCOME(S) ASSESSED, INCLUDE DEPENDENT VARIABLES AND TYPE

L929 fibroblasts changes and ALP activity of Saos-2 after treatment with solutions.

METHOD USED TO ASSESS THE OUTCOMES AND WHO ASSESSED THE OUTCOMES

Cell metabolism was assessed by methyl-thiazole-tetrazolium (MTT); lysosome integrity, by neutral red (NR) assay; type of cell death, by flow cytometry (apoptosis/necrosis); cytoskeleton, by actin and α-tubulin fluorescence; and cell ultrastructure, by transmission electron microscopy (TEM). The alkaline phosphatase (ALP) activity induced by solutions was determined in Saos-2 by thymolphthalein release.

RESULTS

$Ca(OCl)_2$ promoted higher cell viability, and a lower percentage of apoptosis and necrosis than NaOCl ($P < 0.05$). $Ca(OCl)_2$ and NaOCl decreased cell metabolism and lysosome integrity, induced the breakdown of microtubules and actin filaments, promoted alterations of rough endoplasmic reticulum and disruption of mitochondrial cristae, and did not induce ALP activity.

CONCLUSION

Although $Ca(OCl)_2$ and NaOCl promoted the same cytotoxicity mechanism and did not stimulate ALP activity, $Ca(OCl)_2$ was less cytotoxic than NaOCl.

FUNDING DETAILS

This study was supported by fellowships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) – Grant no. 2018/24662-6, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

CONFLICT OF INTEREST

The authors assert explicitly that there is no conflict of interest in connection with this study.

Figure 1 *From: Nagendrababu V, Murray PE, Ordinola-Zapata R, Peters OA, Rôças IN, Siqueira JF Jr, Priya E, Jayaraman J, Pulikkotil SJ, Camilleri J, Boutsioukis C, Rossi-Fedele G, Dummer PMH (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontology: a consensus-based development. International Endodontic Journal May 3. doi: 10.1111/iej.13542. https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13542.
For further details visit: http://pride-endodonticguidelines.org/prile

Preparation of the irrigating solutions

The $Ca(OCl)_2$ and NaOCl stock solutions were prepared at approximately 6%. $Ca(OCl)_2$ was prepared by diluting $Ca(OCl)_2$ powder (Êxodo Científica, Sumaré, SP, Brazil) in distilled water (approximately 6% w/v). The resulting solution was agitated for 30 minutes, and then filtered using filter paper. NaOCl was prepared by diluting a 10% NaOCl solution (AraQuímica, Araraquara, SP, Brazil) in distilled water. Both stock solutions were titrated using the iodine/sodium thiosulfate titration method (Vogel 1962) to determine the free available chlorine, and were then stored at 4°C for no more than 20 days, protected from light. The 2.5% $Ca(OCl)_2$ and 2.5% NaOCl solutions were prepared by diluting the titrated stock solutions, and submitting them to another titration to confirm the free available chlorine at 2.5%. Next, 2.5% $Ca(OCl)_2$ and 2.5% NaOCl were diluted serially using a dilution factor of 1.5 in saline (0.9% sodium chloride), and incubated with cells.

Cell culture and treatment protocol

Permanent cultures of L929 mouse fibroblasts (American Type Culture Collection) and human osteoblast-like cells (Saos-2) were cultured with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) (Gibco by Life Technologies, Paisley, UK), 1% penicillin (100 IU mL^{-1}) and 1% streptomycin (100 mg mL^{-1}) (Gibco by Life Technologies), and then incubated at 37°C, 5% CO_2 and 95% relative humidity. The cells were cultured in 6-, 24- or 96-well plates (Corning, Corning, NY, USA), or on Lab-Tek slides (Nalge Nunc, Naperville, IL, USA) containing DMEM with 10% FBS, and incubated for 24 h. The culture medium was removed, and the cells were incubated with the tested solutions and controls for 10 min, and then incubated with DMEM containing 10% FBS for 4 h. Then, all tests were performed. Saline and DMEM were used as negative controls, and 20% DMSO, as the positive control (Viola et al., 2018; Coaguila-Llerena et al., 2020).

Cell viability by methyl-thiazole-tetrazolium (MTT) and neutral red (NR) assays

The L929 fibroblasts (1.5×10^4 cells $well^{-1}$) were cultivated in 96-well plates for both MTT and NR assays. In the MTT assay, the cell treatment protocol was performed with the tested solutions from 0.0013% - 0.0225% and the controls, after which the culture medium was removed, and 100 μL of 1.0 mg mL^{-1} MTT solution

(Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The cells were incubated in an atmosphere consisting of 5% CO_2 and 95% humidity at 37°C for 3 h. Afterwards, the solution was removed, and 100 µL of acidified isopropyl alcohol (HCl: 0.04N isopropyl alcohol) was added. The optical densities of the solutions were measured using a spectrophotometer (Spectramax M, Molecular Devices, San Jose, CA, USA) at 570 nm wavelength, and analysed using dedicated software (SoftMax Pro, Molecular Devices). The percentage of cell viability was calculated based on the absorbance value of the control (saline), considered as 100%.

The NR assay was performed using the cell treatment protocol with the tested solutions from 0.0013% - 0.0225% and the controls, after which the culture medium was removed, and 100 µL of 0.05 mg mL^{-1} NR solution (Sigma-Aldrich) was added to each well. The cells were then incubated in an atmosphere consisting of 5% CO_2 and 95% humidity at 37°C for 3 h. Next, 100 µL of 1% acetic acid solution in 50% ethanol was added to each well. The optical densities were measured using a spectrophotometer (Spectramax M, Molecular Devices) at 570 nm wavelength, and analysed using the software belonging to the equipment (SoftMax Pro, Molecular Devices). The percentage of cell viability was calculated using the absorbance value of the control (saline), considered as 100%. The NR and MTT assays were performed in triplicate and repeated at 3 different time points.

Cell death analysis by flow cytometry

The treatment protocol was conducted with the tested solutions at 0.0013%, 0.0019%, 0.0029%, 0.004%, 0.006% and 0.015%, after which L929 fibroblasts (8×10^4 cells $well^{-1}$) previously cultured in 24-well plates were washed with phosphate-buffered saline (PBS), and collected from the culture plate wells using nonenzymatic detergent (Gibco by Life Technologies). Hydrogen peroxide (1 mM) was the positive control. The cells were incubated in buffer solution (1x Binding Buffer) containing Annexin V-FITC and propidium iodide (PI), according to the manufacturer's instructions (eBioscience^{TM} Annexin V-FITC Apoptosis Detection Kit; Invitrogen by Thermo Fisher Scientific, Vienna, Austria). At least 1×10^5 cells per sample were acquired and analysed with a fluorescence-activated cell sorter (FACS; Beckton Dickinson, San Jose, CA, USA). The specific gated individualised populations were evaluated based on size (FSC) and granularity (SSC) or fluorescence (FL) parameters. A total of 20 000 events were analysed for each sample. The analysis was performed according to the following parameters: viable cells (FITC-/PI-), initial apoptosis (FITC+/PI-), final apoptosis (FITC+/PI+), and cell necrosis (FITC-/PI+). The results were presented as viable cells, apoptosis and necrosis (final apoptosis + cell necrosis). The experiments were performed in triplicate and repeated at 2 different time points.

Cytoskeleton analysis by actin and α-tubulin fluorescence

L929 fibroblasts (6 x 10^4 cells $well^{-1}$) were cultured on Lab-Tek^{TM} slides (Nalge Nunc, Rochester, New York, USA), and subjected to the treatment protocol with tested solutions at 0.0029%, 0.006% and 0.015%. The cells were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes, washed 3 times with PBS, and permeabilised with 0.5% triton x-100 (Sigma-Aldrich). The microtubules were labelled with α-tubulin antibody (mouse monoclonal anti-α-tubulin; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) at 4°C overnight, and then with FITC-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA; 1:200) at 37°C for 1 h. The actin filaments were labelled by incubating the cells with actin (Alexa Fluor® 594 Phalloidin - Molecular Probes, Eugene, OR, USA) diluted in 1% bovine serum albumin (BSA), 1:100, for 30 minutes at 37°C. DNA labelling consisted of incubating the cells with 300 nM DAPI (4', 6-diamidino-2-phenylindol, Sigma-Aldrich) for 3 minutes at 37°C. The slides were mounted with Prolong^{TM} (Invitrogen Molecular Probes, Eugene, Oregon, USA). The cells were analysed and photographed using a Leica DM 6000M microscope (Leica Microsystems, Wetzlar, Germany) coupled to a Leica AF6000 Deconvolution System (Leica Microsystems).

Analysis of cell ultrastructure by transmission electron microscopy (TEM)

This detailed morphological evaluation was performed to identify the ultrastructural alterations induced by irrigating solutions at 0.0029%, 0.006% and 0.015% in L929 fibroblasts (10 x 10^5 cells $well^{-1}$, cultured in 6-well plates). The procedures were performed according to a previous study (Faria et al., 2009). Briefly, after application of the treatment protocol, the cells were removed from each well using a scraper (Corning), and were centrifuged at 400 G for 6 minutes at 20º C. Then, they were fixed in 1.4% glutaric aldehyde in 0.1 mol L^{-1} sodium cacodylate buffer with sucrose 0.2 mol L^{-1} (pH 7.4) at 37°C for 30 min, and post-fixed in 1% osmium tetroxide at 4°C for 1 hour. The cells were washed in 0.1 mol L^{-1} sodium cacodylate buffer, and then kept in a solution of 0.5% uranyl acetate with sucrose for

12 hours at 4°C (block contrast). Next, the cells were dehydrated in ascending concentrations of acetone, and embedded in Araldite® 502 resin (Polysciences, Warrington, PA, USA). Ultrathin sections were observed and photographed using a transmission electron microscope (EM 109, Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 80 kV.

Alkaline phosphatase (ALP) activity

Saos-2 were cultured in 96-well plates at 1 x 10^4 cells well^{-1} and incubated for 24 h. Then, they were incubated with $Ca(OCl)_2$ and NaOCl solutions at 0.002%, 0.0002%, 0.00002% and 0.000002% for 1, 3 and 7 days. The MTT assay was performed after each period. The 0.00002% and 0.000002% concentrations showed no cytotoxic effects up to 7 days (data not shown); for this reason, the 0.00002% was chosen to assess ALP activity. The cells were incubated with 0.00002% $Ca(OCl)_2$ and NaOCl for 1, 3 and 7 days. After each period, the cells were incubated with a sodium lauryl sulphate solution (1 mg mL^{-1}) at room temperature for 30 minutes, and the commercial kit solution (Labtest, Lagoa Santa, MG, Brazil) was added following the manufacturer's instructions. The optical density of the solutions was measured by a spectrophotometer (Spectramax M, Molecular Devices) at 590 nm filter, and the ALP activity was calculated as µmol thymolphthalein / min / L / OD. The data were expressed as ALP activity normalised by the total protein content, which was also determined with the Labtest kit. A simultaneous MTT assay was performed at 1, 3 and 7 days to monitor cell viability (Ochoa-Rodríguez et al., 2019).

Statistical analysis

The data were analysed using the Graph Pad Prism 5 statistical program (GraphPad Software, La Jolla, CA, USA), using a significance level of 5%. The statistical tests used were two-way ANOVA and Bonferroni's post-test.

Results

Cell viability

The results are shown in Figure 2. $Ca(OCl)_2$ and NaOCl had an effect on cell viability in a concentration dependent manner, i.e., the higher the concentration, the higher the cytotoxicity. The MTT assay revealed that $Ca(OCl)_2$ promoted higher cell viability than NaOCl at the intermediate concentrations of 0.0029%, 0.004% and 0.006% ($P < 0.05$). The NR assay showed that $Ca(OCl)_2$ promoted higher cell viability at the lowest concentrations of 0.0013% and 0.0019% ($P < 0.05$).

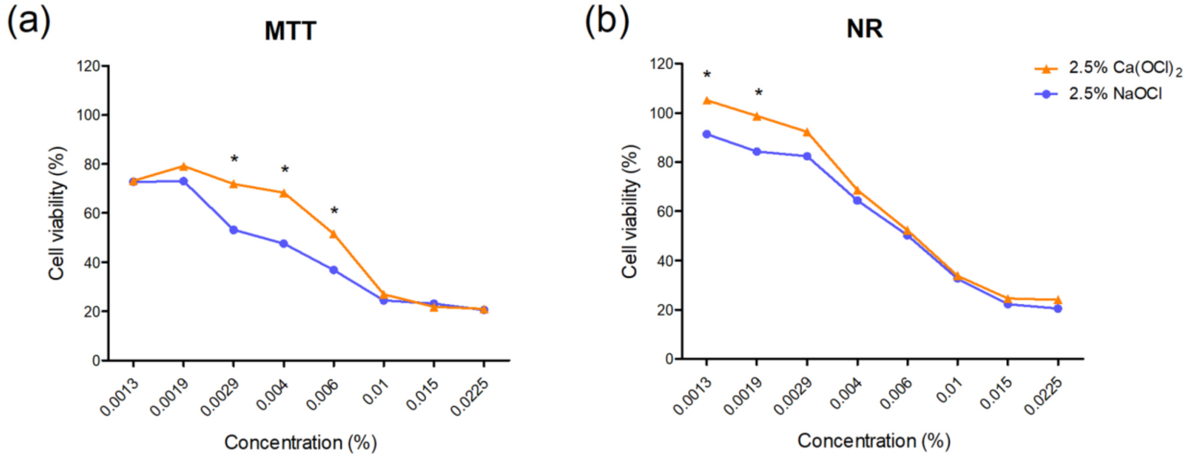

Figure 2 Viability of L929 fibroblasts after exposure to 2.5% calcium hypochlorite [$Ca(OCl)_2$] and 2.5% sodium hypochlorite (NaOCl) solutions at different concentrations by (a) MTT and (b) NR assays. (*) Represents significant differences between the solutions at the same concentration ($P < 0.05$). MTT, methyl-thiazole-tetrazolium; NR, neutral red.

Cell death

The results are shown in Figure 3. At lower concentrations (0.0013%, 0.0019%, 0.0029%), there was no difference among $Ca(OCl)_2$, NaOCl, and the control regarding the percentage of viable cells ($P > 0.05$). At 0.004%, both $Ca(OCl)_2$ and NaOCl had a lower percentage of viable cells than the control group ($P < 0.05$), and there was no difference between the solutions ($P > 0.05$). At higher concentrations (0.006% and 0.015%), $Ca(OCl)_2$ showed a higher percentage of viable cells than NaOCl ($P < 0.05$). $Ca(OCl)_2$ had a lower percentage of apoptotic cells than NaOCl ($P < 0.05$), except at 0.0029% and 0.015% ($P > 0.05$). Regarding necrosis, $Ca(OCl)_2$ had a lower percentage of necrosis than NaOCl at 0.006% and 0.015% ($P < 0.05$).

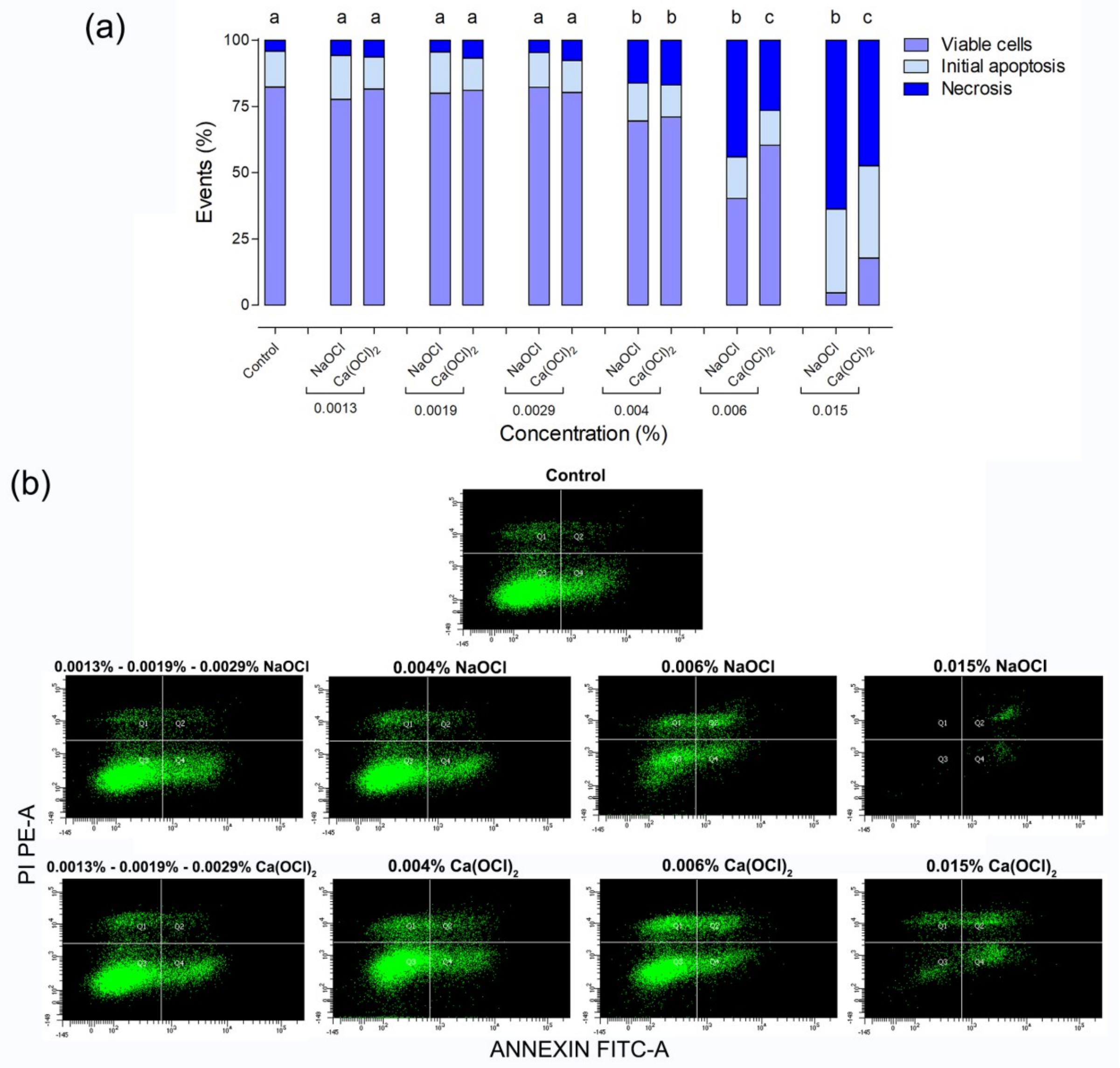

Figure 3 Cell death analysis of L929 fibroblasts by flow cytometry. (a) Percentage of viable cells, initial apoptosis or necrosis after exposure to calcium hypochlorite [$Ca(OCl)_2$], sodium hypochlorite (NaOCl) and control (saline) at different concentrations. Different letters in each 2-column grouping (in each concentration) and control group represent a significant difference among the groups, regarding viable cells. (b) Representative scatter plot of the control, $Ca(OCl)_2$ and NaOCl at different concentrations. The populations of viable (FITC-/PI-), apoptotic (FITC+/PI-) and necrotic (FITC+/PI+ and FITC-/PI+) cells are represented in the left inferior, right inferior and left superior + right superior quadrants, respectively.

Cytoskeleton analysis

The L929 fibroblasts exposed to the controls (saline and DMEM) showed a regular distribution of microtubules (α-tubulin) and actin filaments around the nuclei, indicating that the entire cytoplasmic volume was occupied. Both $Ca(OCl)_2$ and NaOCl had a similar effect on the microtubules and actin filament. There were changes in the configuration of the microtubules and actin filaments at 0.006%, evidenced by cell condensation of microtubules and actin filaments, roundness and decreased size. The changes were more pronounced for NaOCl. At 0.015%, both $Ca(OCl)_2$ and NaOCl promoted microtubules, actin filaments and nucleus collapse, typical signs of cell necrosis (Figures 4 and 5).

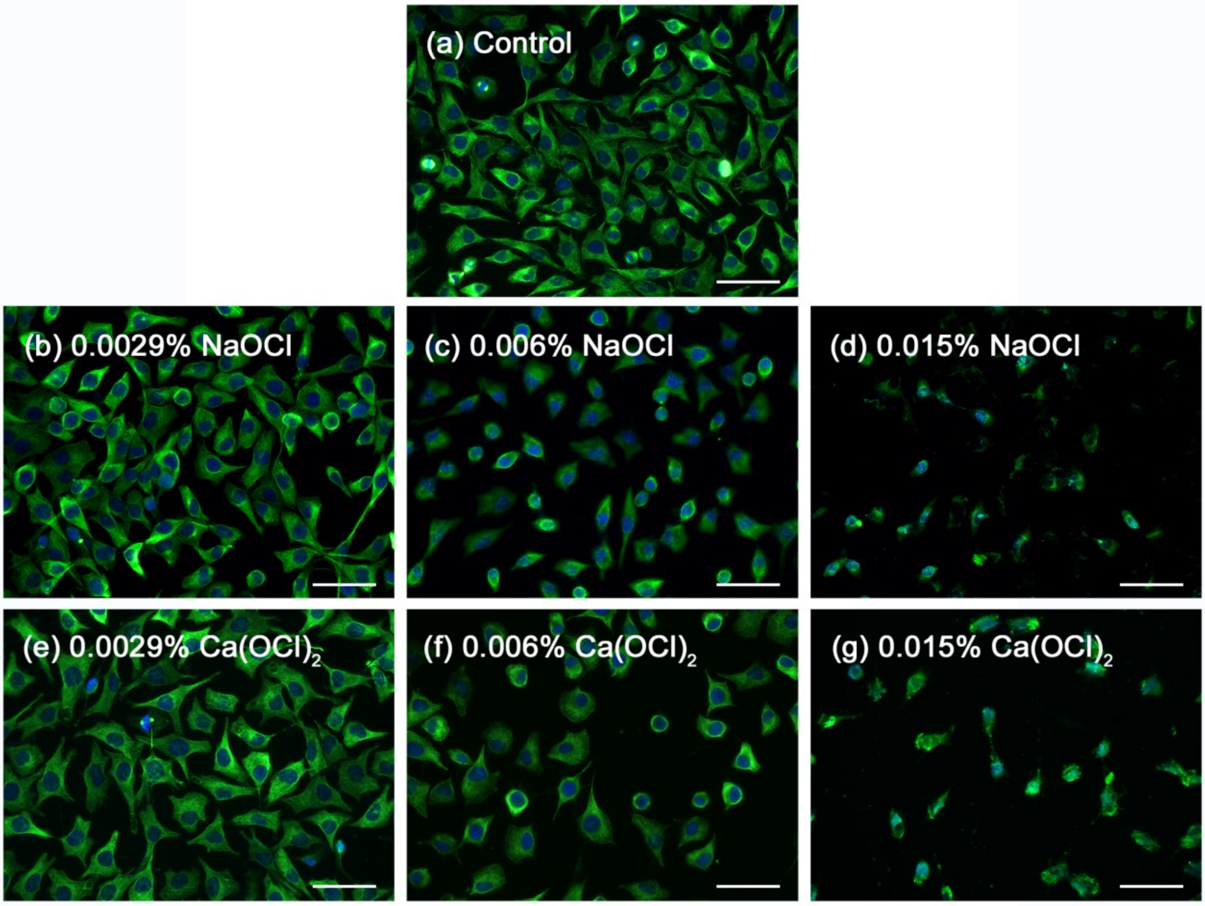

Figure 4 Cytoskeleton analysis by α-tubulin immunofluorescence of L929 after exposure to solutions at 0.0029%, 0.006% and 0.015% (green fluorescence for α-tubulin, and blue fluorescence for nuclei). Control: saline and culture medium (a), sodium hypochlorite – NaOCl (b–d), calcium hypochlorite – $Ca(OCl)_2$ (e–g). Magnification 40x, bar = 50 µm.

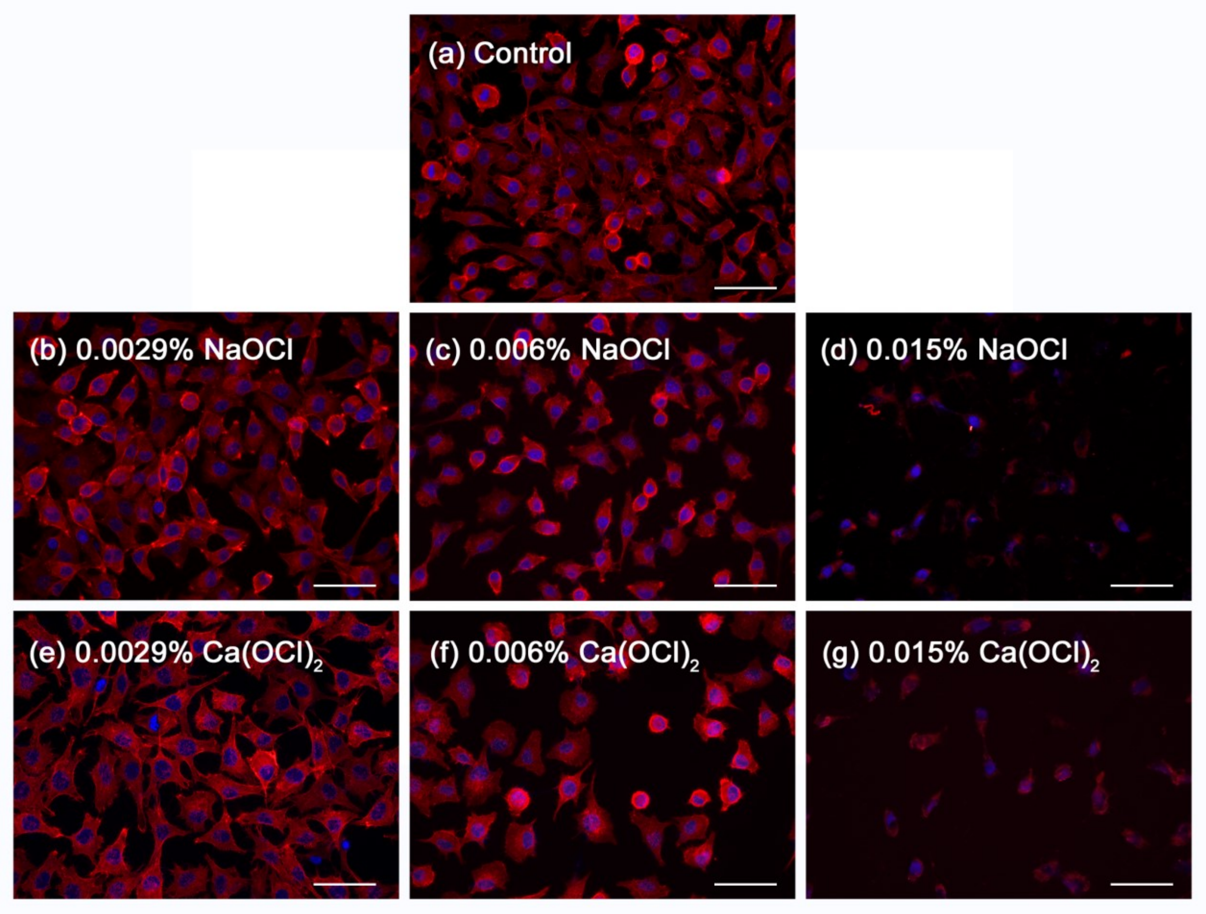

Figure 5 Cytoskeleton analysis by actin immunofluorescence of L929 fibroblasts after exposure to solutions at 0.0029%, 0.006% and 0.015% (red fluorescence for actin, and blue fluorescence for nuclei). Control: saline and culture medium (a), sodium hypochlorite – NaOCl (b–d), calcium hypochlorite – $Ca(OCl)_2$ (e–g). Magnification 40x, bar = 50 µm.

Cell ultrastructure

There was a progressive degradation of the ultrastructure of the L929 fibroblasts directly proportional to the increase in dose of both NaOCl and $Ca(OCl)_2$ (Figure 6). At 0.0029%, both solutions showed a structure similar to the control; however, the mitochondrial cristae in the NaOCl group began to disrupt. At 0.006%, both NaOCl and $Ca(OCl)_2$ revealed that the rough endoplasmic reticulum had become round, was electrodense, and showed protein accumulation. Additionally, more apparent empty vacuoles were observed for NaOCl. At 0.015%, both solutions showed L929 fibroblasts with cellular component degradation, membrane fragmentation, and cytoplasmic content collapse, typical signs of cell necrosis.

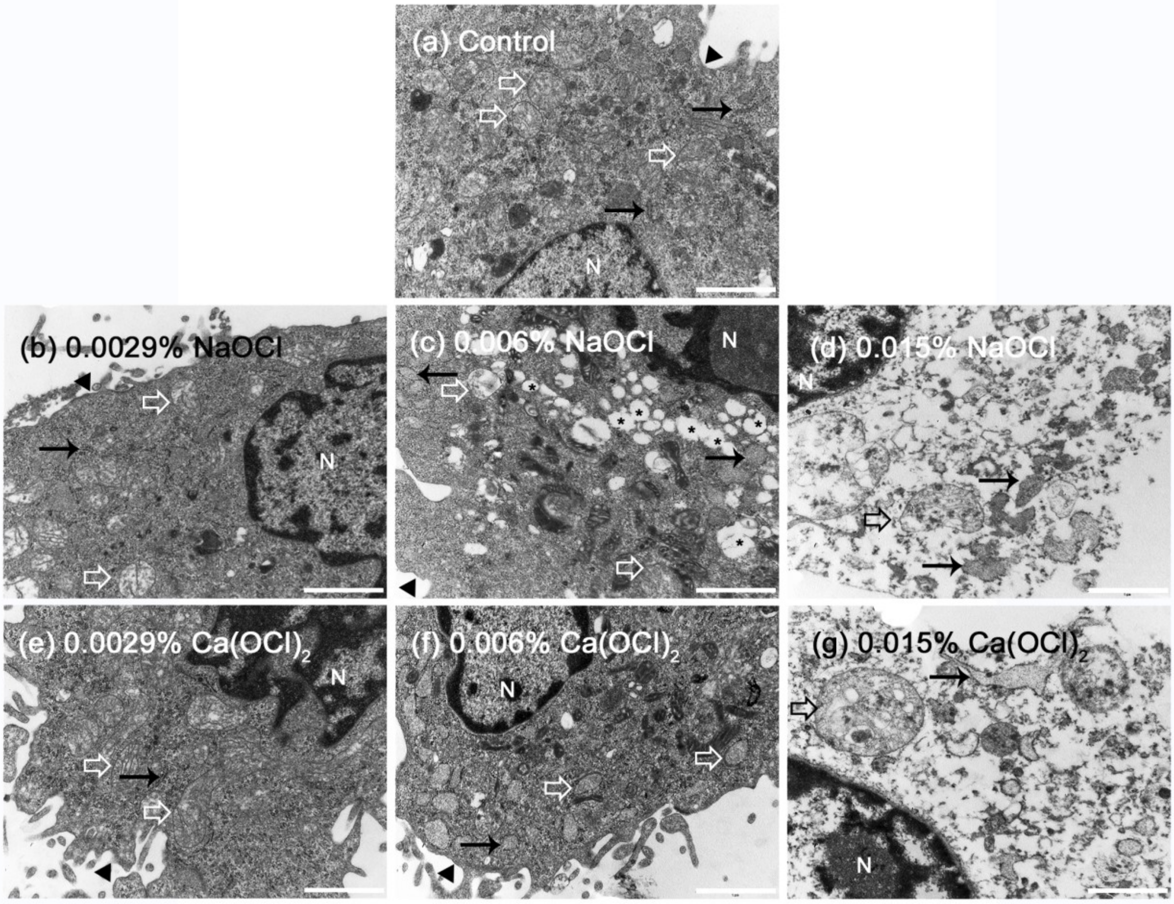

Figure 6. Representative images by transmission electron microscopy of L929 fibroblasts after exposure to solutions at 0.0029%, 0.006% and 0.015%. Control: saline and culture medium (a), sodium hypochlorite – NaOCl (b–d), calcium hypochlorite – $Ca(OCl)_2$ (e–g). Black head filled arrow, cell membrane; empty arrow, mitochondria; N, nuclei; black filled arrow, rough endoplasmic reticulum; and *, vacuole. Bar = 1 μm.

ALP activity

The MTT assay (Figure 7a) revealed that there was no difference in the viability of the Saos-2 cells exposed to NaOCl, $Ca(OCl)_2$ and the control (culture medium) at any of the time points ($P > 0.05$). Figure 7b shows that NaOCl and $Ca(OCl)_2$ inhibited ALP activity at 1 and 3 days, and that there was no difference among all groups at 7 days ($P > 0.05$).

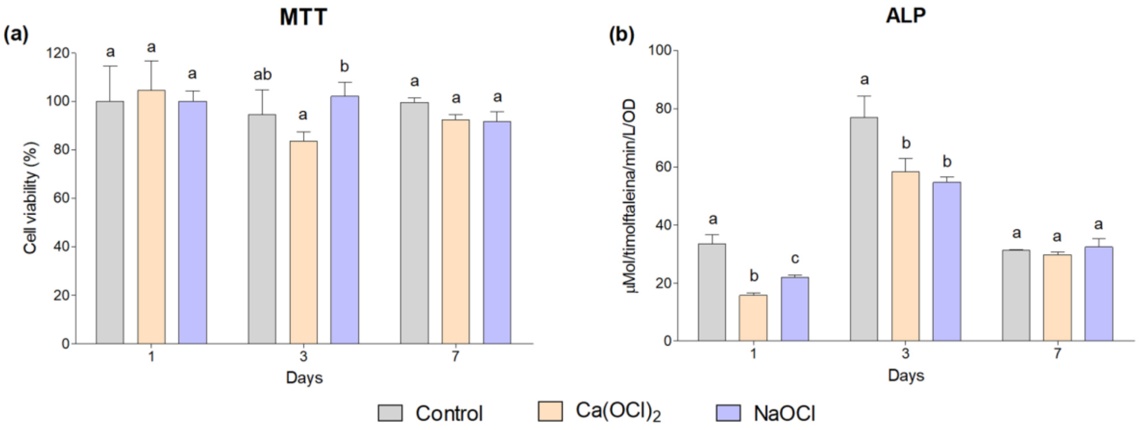

Figure 7 (a) Viability of Saos-2 by methyl-thiazole-tetrazolium (MTT) assay and (b) alkaline phosphatase (ALP) activity after exposure to calcium hypochlorite [$Ca(OCl)_2$], sodium hypochlorite (NaOCl) and culture medium (control) for 1, 3 and 7 days at 0.00002%. Different letters at each period indicate significant differences among the solutions.

Discussion

Although the antimicrobial properties of endodontic irrigants are important, toxicity is a matter of concern, since a toxic irrigant may create suboptimal conditions for cells to grow, attach, and differentiate (Alfadda et al., 2021). Ideally, an optimum endodontic irrigant should have minimal or no toxicity to avoid any damage to the periapical tissues (Bapat et al., 2021). The present study aimed to characterise the injury in L929 fibroblasts induced by 2.5% $Ca(OCl)_2$, as well as determine the effect of this solution on the biology of Saos-2, compared to 2.5% NaOCl. The null hypothesis was rejected because differences were founded between the solutions.

In vitro systems are used to provide information on the mechanisms of toxicity and to reduce both the use of laboratory animals and the complexity involved in *in vivo* systems (Hidalgo & Domínguez 1998). The mechanism of NaOCl cytotoxicity has been previously reported (Viola et al., 2018). In the case of $Ca(OCl)_2$, there is no evidence of any such mechanism. In the present study, L929 fibroblasts were exposed to tested solutions for 10 minutes to simulate the approximate time needed for root canal instrumentation (Lotfi et al., 2012). L929 fibroblasts were used because they are recommended by an ISO standard (ISO 10993-5 2009), and because they are used to assess the cytotoxicity of endodontic irrigants (Viola et al., 2018; Coaguila-Llerena et al*.*, 2019; Coaguila-Llerena et al., 2020).

Different cellular parameters can be evaluated in such a way as to enable a more reliable interpretation of cytotoxic effects (Fotakis & Timbrell 2006). The MTT assay allows the activity of mitochondrial succinate dehydrogenases to be evaluated through the quantity of formazan crystals generated by living cells that metabolise the tetrazolium salt (Van Tonder et al., 2015). The NR assay assesses membrane permeability and lysosomal activity/integrity (Ates et al., 2017). NR, a weak cationic dye, penetrates the cell membrane at a physiological pH, and binds in the lysosomes. The proton gradient inside the lysosomes ensures a more acidic pH, and the dye becomes charged and is retained. When the cell dies or the pH gradient is reduced, the dye is no longer retained. Thus, the amount of retained NR can be correlated to the number of viable cells (Repetto et al., 2008). Both MTT and NR revealed that the 2.5% $Ca(OCl)_2$ group had higher cell viability than the 2.5% NaOCl group; that is, $Ca(OCl)_2$ had fewer effects on metabolism and lysosomal integrity than NaOCl. This lower cytotoxicity of $Ca(OCl)_2$ has been previously observed in the same cell line (Sedigh-Shams et al*.,* 2016; Coaguila-Llerena et al., 2019). Speculatively, Ca(OCl)2, an oxidising agent, has a lower cytotoxic effect, because chlorine is released more slowly in it than in NaOCl (Sedigh-Shams et al., 2016). On the other hand, previous studies have shown that $Ca(OCl)_2$ promoted cytotoxicity similar to or higher than NaOCl in 3T3 fibroblasts (Blattes et al., 2017; Yilmaz et al., 2020). The differences may be explained by the non-determination of the free available chlorine of both $Ca(OCl)_2$ and NaOCl before the experimental procedures, and the consequent use of these solutions in concentrations different from those expected. NaOCl concentrations are not always accurate due to their unstable nature (Leonardo et al., 2016). Although it has been reported that preparation of $Ca(OCl)_2$ solution from its powder may provide a more accurate value of free available chlorine than NaOCl (Leonardo et al., 2016), we observed that this value may not always be the expected amount, and may vary more or less, depending on the commercial product or the expiration date (data not shown). Considering that the cytotoxicity of the chlorinated solutions is directly related to their free available chlorine value, which is associated to their oxidant capacity (Than et al., 2003; Viola et al., 2018; Coaguila-Llerena et al., 2020), titration becomes essential.

Cells have a great capacity to recover from an attack by adapting to the changes that surround them; however, if the injury is too severe, cell death can occur

(Fitzpatrick & Gordon 2018). The main forms of cell death include apoptosis and necrosis (Vanden Berghe et al., 2013). Necrosis is a process of cytoplasmic swelling, dilation of organelles, vacuolation, rupture of membranes and leakage of cytoplasmic content (Banfalvi 2017). Apoptosis is a programmed type of cell death characterised by cell shrinkage, plasma membrane blebbing, cell detachment, nuclear condensation, DNA fragmentation, externalisation of phosphatidylserine and activation of caspases (Henry et al., 2013). If an endodontic material primarily induces apoptosis, then tissue damage is limited, whereas if it induces necrosis, the healing is retarded (Ciapetti et al., 2000). Both $Ca(OCl)_2$ and NaOCl induced necrosis and apoptosis; however, $Ca(OCl)_2$ induced a lower percentage of both. A previous study showed that NaOCl predominantly induced necrosis (Viola et al., 2018). Its authors used a higher concentrations of NaOCl, which may explain the differences, since the higher the disinfectant concentration, the higher the percentage of cell death by necrosis (Faria et al., 2007). It is important to note that, although we use the term “necrosis” for the results of flow cytometry, PI alone discriminates dead cells that have permeable plasma membranes, regardless of the mechanism of death; that is, it does not necessarily represents necrosis (Crowley et al., 2016). According to the available literature, the identification of necrosis should not be based solely on morphological or biochemical criteria, but should include different methods (Krysko et al., 2008; Vanden Berghe et al., 2013). For this reason, necrosis was confirmed by cytoskeleton and cellular ultrastructure analyses. It is important to emphasise that when apoptosis is assessed in cultured cells, incubation time periods higher than four to six hours should be avoided, since apoptotic cells are not phagocyted in the culture, and can hence initiate secondary necrosis from apoptosis (Vanden Berghe et al., 2013). For this reason, a 4-hour incubation time of the cells in contact with the culture medium was applied after removal of the tested solution (Viola et al., 2018; Coaguila-Llerena et al., 2020).

The inside of a cell contains an organised, well-regulated network called the cytoskeleton, which contains microfilaments, microtubules, and intermediate filaments. This network is involved in processes such as endocytosis, cell division, intra-cellular transport, motility, reaction to external forces, and adhesion, among others (Binderman et al., 2014; Hohmann & Dehghani 2019). Given that the cytoskeleton is key to virtually all physiological cellular processes, abnormalities in this essential cellular feature can lead to disease and death (Binderman et al., 2014). In the present study, changes in the cytoskeleton were proportional to the solution concentrations, and were similar for both $Ca(OCl)_2$ and NaOCl; however, they were slightly less marked for $Ca(OCl)_2$. At the highest concentration, a rupture of the cytoskeleton and nucleus was observed, a characteristic feature of cell necrosis. This effect was observed for NaOCl in a previous study (Viola et al., 2018).

Transmission electron microscopy (TEM) is the most powerful morphological method to describe the ultrastructural changes in physiological and pathological conditions occurring inside cells and organelles (Burattini & Falcieri 2013). Both solutions (NaOCl and $Ca(OCl)_2$) induced an ultrastructure degradation directly proportional to the dose. This is in line with a previous study dealing with NaOCl, which used the same L929 cell line, but at different concentrations (Viola et al*.,* 2018). At intermediate concentrations, both solutions induced protein accumulation in the rough endoplasmic reticulum (Kroemer & Reed 2000). Additionally, empty vacuoles were observed in the NaOCl group, corroborating the findings of a previous study (Viola et al., 2018).

$Ca(OCl)_2$ dissociates in water to form hypochlorous acid and calcium hydroxide (Dutta & Saunders 2012). It was hypothesised that the calcium ions inherent in calcium hydroxide could promote some type of osteogenic induction and subsequent mineralisation. The ability of an endodontic material to induce mineralisation can be assessed based on the activity of ALP (Modareszadeh et al., 2012). Saos-2 cells were used because they have an osteoblastic phenotype and express high levels of ALP activity (Rodan et al., 1987; Modareszadeh et al., 2012). The concentration chosen was 0.00002%, because the pilot cell viability test (MTT) indicated that no cytotoxic effects were observed in the solutions after 7 days of exposure. It is important to use non-cytotoxic concentrations for this assay, since only viable cells have ALP activity (Coaguila-Llerena et al., 2020). Neither $Ca(OCl)_2$ nor NaOCl induced ALP activity of Saos-2, in agreement with studies that showed the same effect in NaOCl using Saos-2 (Coaguila-Llerena et al., 2020), and $Ca(OCl)_2$ using dental pulp stem cells (Alfadda et al. 2021). Regarding $Ca(OCl)_2$, the results are also in line with previous studies showing that calcium ions inhibited ALP activity in human dental cells (An et al., 2012; Li et al., 2015). However, these studies indicated that calcium ions promoted osteogenic/odontogenic differentiation and mineralisation of human dental pulp cells, as demonstrated by the expression levels of genes, such as osteopontin, osteocalcin, and DSPP (dentin sialophosphoprotein), and by the production of mineralised nodules. Thus, further research is needed to evaluate parameters such as these in cells exposed to $Ca(OCl)_2$.

It is worthwhile noting that although *in vitro* tests are useful for understanding the mechanisms of cytotoxicity, they have limitations, such as not presenting physiological conditions, being deficient in evaluating cellular interactions, and not including biotransformation and defence mechanisms (Hartung & Daston 2009).

Conclusion

Although $Ca(OCl)_2$ and NaOCl promoted the same cytotoxicity mechanism, $Ca(OCl)_2$ was less cytotoxic than NaOCl. Both solutions led to a reduction in cell metabolism and lysosomal activity/integrity, cell death induction by apoptosis and necrosis, cytoskeleton breakdown, and cell ultrastructure degradation, and neither stimulated ALP activity.

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3.3 Publication 3*

OUTCOMES OF THE GENTLEWAVE SYSTEM ON ROOT CANAL TREATMENT: A NARRATIVE REVIEW

Abstract

This study aimed to describe the outcomes of the GentleWave system (GW) (Sonendo) on root canal treatment. Published articles were collected from scientific databases (MEDLINE/PubMed platform, Web of Science, Scopus, Science Direct and Embase). A total of 24 studies were collected from August/2014 to July/2021, 20 *in vitro* and 4 clinical. GW System was not associated with extrusion of the irrigant, promoted faster organic dissolution than conventional syringe irrigation (CSI), passive ultrasonic irrigation (PUI) continuous ultrasonic irrigation (CUI) and EndoVac, reduced more bacterial DNA and biofilm than PUI and CUI, promoted higher penetration of sodium hypochlorite into dentinal tubules than PUI and CUI *in vitro*, and removed more intracanal medication than CSI and PUI. GW was able to remove pulp tissue and calcifications. Moreover, its ability to remove hard-tissue debris and smear layer was better than that of CSI, and its ability to remove root canal obturation residues was lower or similar to that of PUI, and similar to that of CSI and EndoVac. Regarding root canal obturation of minimally instrumented molar canals, GW was associated with high-quality obturation. Clinically, the success rate of endodontic treatment using GW was 97.3%, and the short-term postoperative pain in the GW group was not different from CSI. Further research, mainly clinical, is needed to establish whether GW has any advantages over other available irrigation methods.

Keywords: Disinfection; Endodontics; GentleWave; Multisonic ultracleaning

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[*] This is the peer-reviewed version of the article “Outcomes of the GentleWave system on root canal treatment: a narrative review”, which has been published in final form at [https://rde.ac/search.php?where=aview&id=10.5395/rde.2022.47.e11&code=2185RDE&vmode=FULL] in the *Restorative Dentistry & Endodontics 2022*.

INTRODUCTION

The primary goal of root canal treatment is to eliminate and prevent re-infection of the root canal system [1]. In necrotic teeth, microorganisms can colonize anatomical complexities, such as isthmuses, ramifications and dentinal tubules [2]. Instrumentation can reduce the bacterial load by approximately 80% [3]. However, the microorganisms cannot be completely eliminated, because the instruments are unable to fully reach the anatomical complexities of the root canal system [2]. For this reason, irrigation is the key to successful treatment, since it is the only way to reach areas left untouched by instrumentation [4].

Sodium hypochlorite (NaOCl) is the most widely used solution, owing to its antimicrobial/antibiofilm activity, and organic tissue dissolution capacity [5-7]. Additionally, since NaOCl cannot remove inorganic tissue, 17% ethylenediaminetetraacetic acid (EDTA) is recommended to address this limitation [8].

Traditionally, the irrigant is delivered to the root canal through a needle coupled to a syringe, a system known as conventional syringe irrigation (CSI) [9]. CSI has a rinsing effect, which is an important part of the irrigation process [10]. However, it does not guarantee that the irrigant will reach the working length and anatomical complexities [9,11]. Furthermore, CSI can exert positive pressure that could trigger the extrusion of the irrigant [12]. For this reason, different methods have been introduced to improve the safety and cleaning of the root canal system, such as negative pressure irrigation (EndoVac), and passive ultrasonic irrigation (PUI) [13,14].

The GentleWave system (GW) (Sonendo, Laguna Hills, CA, USA) was introduced on the US market in 2014, and represents a type of endodontic device developed for cleaning and disinfection of the root canal [15]. According to its manufacturer, GW can be used in situations that need only minimal instrumentation, instead of using conventional instrumentation, which may contribute to maintain tooth resistance [16,17]. That is because the widening of the root canal can lower the fracture resistance of the root; however, there is still no consensus that minimal instrumentation has this effect [18,19].

GW creates a powerful, high speed shear force that dispenses irrigants into the root canal system, without having to place the tip of the handpiece into the canal orifice [20,21]. Specifically, the implosion of microbubbles creates an acoustic field of broadband frequencies that travel through the fluid to reach the entire root canal system, thereby cleaning the soft tissues, and eliminating the bacteria within the root canals [20,21]. Additionally, GW provides negative pressure irrigation, which ensures less apical extrusion of the irrigant [12,22]. The equipment can be used with NaOCl, EDTA and distilled water [17]. The irrigant is dispensed from the handpiece, coupled to the tooth, at a speed of 45 mL/min [23], and the excess irrigant is removed concurrently [22].

Currently, GW costs approximately $80,000.00 per console, and $50.00 to $100.00 for a one-time use handpiece. However, several doubts have been raised in regard to GW: Is it worth investing in such high-cost equipment? Does it produce better results than conventional root canal treatment? What are the effects of GW on endodontic treatment? Therefore, the present literature review addressed the scientific evidence on the use of the GW, providing a detailed description of *in vitro* and clinical studies.

REVIEW

Electronic search

The electronic search was performed on MEDLINE (PubMed platform), Web of Science, Scopus, Science Direct and Embase databases. The search string was (“Gentlewave” OR “Multisonic Ultracleaning”) AND (“endodontics” OR “root canal treatment”). The last search was conducted in July 2021. In addition, a manual search was performed in Google Scholar, and the following journals: *Journal of Dentistry*, *Journal of Endodontics*, *International Endodontic Journal*, *Clinical Oral Investigations*, *Restorative Dentistry & Endodontics*, and *Australian Endodontic Journal*. The language was restricted to English.

A total of 24 studies from August/2014 to July/2021 were collected (20 *in vitro* and 4 clinical-focused), and all focused on relating the effects of GW on root canal treatment (Table 1).

Study	Year of study	Type of study	Parameter	Investigative criteria	Results
Sigurdsson *et al.* [30]	2016	Clinical	Success rate (6 mon)	Clinical signs/symptoms and periapical index scores	GW showed 97.4% success rate
Sigurdsson *et al.* [32]	2016	Clinical	Success rate (12 mon)	Clinical signs/symptoms and periapical index scores	GW showed 97.3% success rate
Sigurdsson *et al.* [21]	2018	Clinical	Healing of periapical lesion (12 mon)	Clinical signs/symptoms and periapical index scores	GW showed 97.7% success rate
Grigsby *et al.* [41]	2020	Clinical	Post-operative pain (up to 168 hr)	Numeric rating scale	No difference between GW and CSI+PUI
Haapasalo *et al.* [23]	2016	*In vitro*	Apical pressure	Specific measurement setup	GW produced negative pressure, while CSI produced positive pressure
Ordinola-Zapata *et al.* [12]	2021	*In vitro*	Apical pressure	Specific measurement setup	GW produced negative pressure, while CSI (open-ended and side-vented) produced positive pressure
Charara *et al.* [22]	2016	*In vitro*	Apical extrusion	Specific measurement setup	GW did not produce apical extrusion
Haapasalo *et al.* [15]	2014	*In vitro*	Tissue dissolution	Mass measurement	GW was faster than PUI, CUI, Endovac and CSI
Chan *et al.* [20]	2019	*In vitro*	Removal of hard-tissue debris	Micro-CT	GW was better than CUI, and not different to PUI
Molina *et al.* [24]	2015	*In vitro*	Root canal debridement (pulpal tissue and dentinal mud)	Histologic	GW was better than CSI
Wohlgemuth *et al.* [25]	2015	*In vitro*	Removal of separated instruments	Radiographic	GW is able to remove separated instruments
Wright *et al.* [40]	2019	*In vitro*	Retreatment (removal of gutta-	Micro-CT	GW was not different to Endovac and CSI

			percha/sealer)		
Crozeta *et al*. [26]	2020	*In vitro*	Retreatment of oval-shaped canals (removal of gutta-percha/sealer)	Micro-CT	PUI was better than GW
Zhang *et al.* [27]	2019	*In vitro*	Disinfection of multispecies biofilm	Real-time PCR and bacterial cultures	GW promoted higher reduction of total microbial DNA than CUI
Choi *et al*. [35]	2019	*In vitro*	Biofilm removal	Histologic	GW was better than PUI
Wang *et al*. [28]	2016	*In vitro*	Dentin erosion	SEM and EDS	GW promoted minimal dentin erosion and insignificant changes on dentin composition
Wang *et al.* [34]	2018	*In vitro*	Effects on uninstrumented root canal dentin	SEM	GW promoted no organic tissue remnants or dentin debris
Ma *et al.* [29]	2015	*In vitro*	Removal of $Ca(OH)_2$	Micro-CT	GW as better than PUI and CSI
Liu *et al*. [39]	2022	*In vitro*	Removal of $Ca(OH)_2$ with or without barium sulfate	Assessment of isthmuses of 3D printed canals	GW was faster than CUI
Vandrangi [31]	2016	*In vitro*	Penetration of NaOCl into dentinal tubules	Cristal violet discoloration	GW was better than PUI and CUI
Chen *et al*. [33]	2020	*In vitro*	Removal of calcifications	Micro-CT	GW was able to remove calcifications
Zhong *et al.* [36]	2019	*In vitro*	Removal of hard-tissue debris and effect on obturation	Micro-CT	GW was associated to 93.7% hard-tissue removal, and high quality obturations
Park *et al*. [38]	2020	*In vitro*	Removal of smear layer and obturation residues during retreatment	SEM	GW was not different to PUI and sonic activation
Dash *et al*. [37]	2020	*In vitro*	Removal of smear layer and hard-tissue debris	SEM	GW was better than CSI using 17% EDTA (smear layer and hard-tissue debris) or 5.25% NaOCl (smear layer)

Table 1. Summary of in vitro and clinical studies that used the GentleWave system on root canal treatment. $Ca(OH)_2$, calcium hydroxide; CSI, conventional syringe irrigation; CUI, continuous ultrasonic irrigation; EDS, energy dispersive X-ray spectroscopy; EDTA, Ethylenediaminetetraacetic acid; Micro-CT, microcomputed tomography; NaOCl, sodium hypochlorite; PCR, polymerase chain reaction; PUI, passive ultrasonic irrigation; SEM, scanning electron microscopy; GW, GentleWave system.

1. The GentleWave system

The GW system consists of a console, a handpiece similar to a conventional dental handpiece, and a trash container. A prerequisite for using GW is making sure that the pulp chamber is sealed to prevent communication with the oral cavity, thus preventing NaOCl mist from spreading to the work area. The handpiece tip should be positioned 1mm above the pulp chamber floor when used, to ensure that it does not enter the canal orifices. A touchscreen control panel on the console allows high speed flow regulation of the irrigant to the handpiece, where the irrigant strikes a metal strike plate at the end of the tip, thereby triggering the release of a spray from the tip [15]. The irrigants undergo a degassing process to eliminate the dissolved gas present in the solution, thus optimizing the energy supply through the root canal, and eliminating the vapor-lock effect [16]. When the solution passes from the handpiece into stagnant fluids in the pulp chamber, hydrodynamic cavitation is triggered by shear forces, forming thousands of microbubbles called cavitation clouds. These bubbles implode and create sound waves that cover a wide spectrum of frequencies (Multisonic Ultracleaning spectra) and reverberate throughout the root canal system to achieve more thorough cleaning [24,25]. The handpiece has a 5-point vented suction system that collects excess NaOCl from the pulp chamber [15]. Usually, the protocol is 3% NaOCl for 3 or 5 minutes, followed by rinsing with water for 15 or 30 seconds, 8% EDTA for 2 minutes, and a final rinse with distilled water for 15 or 30 seconds [24-29]. The irrigation flow is 45 mL/min; in other words, 3 or 5 minutes of irrigation with NaOCl is equivalent to 135 mL or 225 mL [15].

2. Root canal instrumentation for the GentleWave procedure

The GW is designed to reduce the need for enlarged instrumentation by applying a minimally invasive endodontic technique designed to preserve dentin [30]. Irrigation protocols with GW call for minimal instrumentation, which includes the use of small files, usually 15.04, S1 ProTaper file with #17 size tip, F1 ProTaper file (20.07), 20.04 or 20.06 [21,22,24,27,28,30-32, 41]. Furthermore, GW follows a conservative philosophy by recommending its use without cervical preflaring [22]. In fact, depending on the research objectives, there are studies that have used GW in non-instrumented canals [33,34]. However, this does not mean that GW cannot be used in canals submitted to conventional instrumentation, defined as enlargement to a minimum size of 25.06, or else 25.07, 30.04, 40.04 or greater [12,20,34].

3. Apical pressure and apical extrusion

NaOCl extrusion is an accident that can lead to complications, such as pain, swelling, and ecchymosis [42]. The apical pressure exerted by GW has been previously evaluated in maxillary and mandibular molars [12,23]. The first study evaluated the apical pressure generated by GW versus CSI, using 30G open-ended and side-vented needles at 1 and 3 mm of the working length of the palatal and distobuccal canals of maxillary molars. Apical pressure levels were measured after no instrumentation, minimal instrumentation with 15.04, conventional instrumentation up to 40.04, and also after enlargement of the apical foramen to size #40. Irrigation with GW generated negative apical pressure (between −13.07 and −17.19 mm Hg), whereas CSI generated positive pressure (6.46 mmHg for side-vented, and 110.34 mmHg for open-ended). The negative apical pressure of GW was not affected by the size of the instrumentation or by the apical foramen [23]. The second study corroborated these observations. The authors evaluated the apical pressure produced by GW in mesial and distal canals of mandibular molars, compared with CSI using 30G open-ended and side-vented needles. The GW generated negative pressure (−30.79 mm Hg), and the 2 needles generated positive pressure, which was lower for the side-vented needle in mesial canals −0.77 mmHg [12].

The apical extrusion of the irrigant produced by GW was compared with that of CSI using a 30G side-vented needle and EndoVac in mesial and distal canals of mandibular molars. The teeth were instrumented up to different sizes (minimal instrumentation up to 15.04, conventional instrumentation up to file 35.06, and over-instrumentation with the 35.06 file exceeding the working length by 1 mm). There was no apical extrusion in the GW and EndoVac groups. In the CSI group, extrusion ranged between 0.000–1.373 g, and was higher in the distal canals, especially after conventional instrumentation and over-instrumentation [22]. As described in the aforementioned studies, GW produces negative apical pressure, which prevents significant extrusion of the irrigant.

4. Organic dissolution ability

Bovine muscle was exposed to 0.5%, 3% and 6% NaOCl for 5 minutes at 21°C and 40°C using GW, PUI (Piezon Master 700 agitation), and continuous ultrasonic irrigation (CUI; Piezon Master 700 agitation plus irrigation), EndoVac and CSI. GW promoted the fastest dissolution, at a rate of 1.0% per second in 0.5% NaOCl, 2.3%

per second in 3% NaOCl, and 2.9% per second in 6% NaOCl. This rate was significantly greater than all other devices. The authors suggested that some form of physical energy created by GW may be responsible for this rapid tissue-dissolving effect [15].

5. Effects on root canal dentin

Wang *et al.* [34] evaluated the morphology of the dentin of non-instrumented premolars irrigated with GW. The analysis of the scanning electron microscopy (SEM) images showed no remains of organic tissue, biofilm, or debris, and more open dentinal tubules. They concluded that the root canals could be cleaned completely without instrumentation, when using GW. Regarding the effects on dentin erosion and dentin composition, a study using SEM images and energy-dispersive X-ray spectroscopy, respectively, revealed that irrigation with GW (using 3% NaOCl, 8% EDTA and water) caused minimal dentin erosion, and minor or insignificant changes in the relative proportions of carbon, oxygen, calcium, and phosphorus in circumpulpal dentin, similar to the effects of CSI using NaOCl followed by final irrigation with EDTA [28].

6. Removal ability

1) Biofilm

The ability of GW to remove multi-species or *Enterococcus faecalis* (*E. faecalis*) biofilm compared with CUI and PUI has already been assessed [27,35]. In the first study reviewed, GW was compared *in vitro* with CUI (ProUltra PiezoFlow) for removing multispecies oral biofilms from canals using quantitative real-time polymerase chain reaction. Root canals were minimally instrumented up to 15.04 (Vortex Blue) for the GW group, and up to 35.04 for the CUI group. Although both systems demonstrated a highly effective reduction in intracanal bacterial DNA, GW showed a more constant and significantly greater reduction in total microbial DNA, *E. faecalis* DNA and *Streptococcus spp* DNA, compared with CUI [27]. Another study was conducted to evaluate the efficacy of GW and PUI (Piezon Master 700 ESI tip activation) in removing *E. faecalis* biofilm. The teeth were submitted to conventional instrumentation (F2 ProTaper + 35.04 EndoSequence) plus PUI, and minimal instrumentation (15.04, EndoSequence) plus GW. Histological analysis indicated that

GW enabled higher biofilm removal in the main canal and isthmus regions, compared with PUI [35].

2) Pulp tissue

The efficacy of GW in removing debris (a combination of soft pulp tissue and dentinal mud) was assessed histologically in comparison with CSI. The teeth from the "conventional instrumentation + CSI" group were instrumented using .04 taper files (Vortex Blue), depending on the apical size of each specimen, and irrigated with a 30G side-vented needle, whereas the teeth of the GW group were minimally instrumented (15.04, EndoSequence), and irrigated with GW. The GW eliminated 97.2% and 98.1% of debris in the apical and middle thirds of the mesial canals in mandibular molars, and mesiobuccal canals in maxillary molars, respectively, while CSI eliminated 67.8% and 87.3% respectively. In the distal root, debridement was similar in both groups. The authors concluded that GW promoted greater debridement than CSI [24].

3) Calcifications

The ability of GW to remove calcifications of distal canals of non-instrumented mandibular molars was assessed using micro-computed tomography (micro-CT) images. By using GW, the average total volume of the canals increased from 5.11 mm^3 to 5.50 mm^3, and 86.4% of the calcifications were reduced. It was concluded that GW can eliminate the calcifications totally or partially, even without previous root canal instrumentation [33].

4) Hard-tissue debris and smear layer

Although GW proved effective in removing hard-tissue (93.7%) after minimal instrumentation, based on micro-CT assessment [36], a study that used SEM images revealed no differences compared with CSI using 17% EDTA as a final irrigation, with photodynamic therapy (PDT) or with Er:YAG laser irradiation [37]. The exception was in the apical third, where GW removed more hard-tissue debris than CSI using 17% EDTA [37]. Another study, using micro-CT images in mandibular molars instrumented with "Small" and "Primary" files (WaveOne Gold), reported that GW promoted higher removal of hard-tissue debris in root canals (96.4%) and isthmuses (97.9%) than CUI

(80.0% and 88.9%, respectively); however, the results for GW were no different from those of PUI (91.2% and 93.5%, respectively) [20].

Regarding smear layer removal, GW was no different from the ultrasonic systems, PDT or Er:YAG [20,37]; however, GW was better than CSI using 17% EDTA or 5.25% NaOCl as a final irrigation [37]. A study analyzing retreated distal canals of molars reported that GW, a PUI system (ENDOSONIC Blue system coupled to a 17.02 file), and sonic agitation with EDDY tips showed higher smear layer removal than another PUI system (Piezon Master 700 coupled to a ESI 15.02 tip), and the negative control (CSI in non-obturated canals), in the middle third. In the apical third, GW, both PUI systems (ENDOSONIC and Piezon Master 700) and EDDY tips provided higher smear layer removal than the negative control [38].

5) Root canal dressing

Regarding this topic, there are 2 studies available [29,39]. In the first one, mandibular molars were instrumented up to 25.08 (mesial canals, WaveOne Gold) and 40.08 (distal canals, WaveOne Gold), and the root canals were filled with Metapaste (containing calcium hydroxide and barium sulfate). After 7 days, the Metapaste was removed using: 1) GW, 2) conventional instrumentation (using the same WaveOne Gold file) plus CSI, or 3) conventional instrumentation plus PUI. The micro-CT analysis revealed that CSI and PUI did not completely remove $Ca(OH)_2$. In the apical third of the mesial and distal canals, CSI removed 47.82% and 77.68%, PUI removed 61.66% and 88.85%, and GW removed significantly more $Ca(OH)_2$, that is, 100% and 98.78%, respectively [29]. The second study compared CSI (open-ended needle and double-side-vented needle), PUI (EndoUltra, coupled to 15.02 tip), CUI (ProUltra PiezoFlow) and GW in the removal of $Ca(OH)_2$ with and without barium sulfate at different proportions from isthmuses of 3D printed transparent root canals. The authors reported that only GW and CUI removed the pastes completely, being GW faster than CUI (2–3 times) [39]. Considering that only 2 studies are available on this important topic, further research should be conducted to derive a stronger clinical correlation.

6) Root canal obturation residues

GW has been compared with CSI, PUI, EndoVac and sonic irrigation [26,38,40]. Crozeta *et al.* [26] performed a micro-CT assessment, and reported that both GW and PUI significantly reduced the volume of obturation material (gutta-percha and AHPlus sealer) remaining from previously instrumented oval canals (R40, Reciproc). Both GW and PUI played a complementary role after initial retreatment using the R50 file (Reciproc). GW was able to remove approximately 10% of the remaining obturation material from the entire canal, while PUI removed 18%, hence achieving better performance. However, another study that used SEM images to assess the debris (residual filling material, dentinal mud and smear layer) revealed no significant differences among GW, PUI systems (Piezon Master 700 and ENDOSONIC Blue system), sonic agitation using EDDY tips, and the negative control (CSI in non-obturated canals) in distal root canals previously retreated with ProTaper Retreatment and ProTaper files [38]. Moreover, the ability of GW to remove root canal obturation was found to be no different from EndoVac or CSI (using a side-vented needle) in canals whose initial retreatment was performed using a .06 tapered heated plugger and ProFile files (up to 20.04) [40].

7) Separated instruments

A study revealed that GW removed separated stainless steel instruments from the root canal [25]. Briefly, 2.5 mm fragments of #10, #15 and #20 K-type files were placed in the middle and apical thirds of extracted molars. The molars were distributed into 2 groups according to the curvature of the root (< 30º and > 30º) and treated with GW. GW promoted greater fragment removal in the middle third (83%) compared to the apical third (61%). Regarding root curvature, GW was more successful in less curved canals (91%) than in more curved canals (42%). The #10, #15, and #20 K-type file fragments were removed 75%, 92%, and 50%, respectively. Additionally, the mean treatment time to remove the fragment was 10 minutes and 44 seconds.

7. Penetration of NaOCl into dentinal tubules

The penetration of irrigants into dentinal tubules is important because bacterial invasion in teeth that present pulpal necrosis has been previously reported [43]. This aspect of GW was compared with that of PUI (Piezon Master 700 coupled to ESI 15.02 tip), and that of CUI (Piezon Master 700 using ESI tip with maximum irrigation rate) in minimally instrumented molars (15.04, EndoSequence) pre-stained with crystal violet. GW promoted higher penetration depth of NaOCl in the coronal, middle and apical thirds than PUI and CUI. The authors concluded that the penetration promoted by GW was 4 times as deep as that of ultrasonic systems [31].

8. Effect of GW on final obturation

The effect of GW on the final obturation after minimal instrumentation of root canals of maxillary molars (15.04, Vortex Blue) was evaluated using micro-CT images [36]. The root canals were filled using a modified single cone technique with 3 different sealers: GuttaFlow Bioseal, GuttaFlow 2 and MTA Fillapex. The sealers provided an 89.5%–98.9% filled canal, pointing out that GuttaFlow Bioseal (96.9%–98.9%) and GuttaFlow 2 (94.7%–97.5%) were higher than MTA Fillapex (89.4%–89.5%). It was concluded that the modified single cone technique using GuttaFlow 2 and GuttaFlow Bioseal sealers resulted in a high-quality obturation after using GW in minimally instrumented molar canals.

9. Clinical studies

Two of the clinical studies that included GW in the protocol treatment assessed the success rate of endodontic treatment after 3 months, 6 months and 12 months, and a third evaluated healing of the periapical lesion after 12 months [21,30,32]. Regarding the studies reporting the success rate of endodontic treatment using GW, a multicenter prospective study using 89 teeth indicated that the success rate after 3 months was 92% [32]. After 6 months, 77.9% of the vital or necrotic teeth were classified as “healed,” 19.5%, as “healing,” and 2.6%, as “diseased.” In other words, when combining “healed” with “healing,” 97.4% were classified as “successful”. No comparison was made with any other type of treatment protocol. Additionally, the preoperative presence of a periapical lesion (periapical index [PAI] ≥ 3), and a single session were correlated with a “diseased” episode [30]. After 12 months, the success rate was 97.3% [32]. The third study aforementioned resulted from 2 clinical studies. It reported that the healing of periapical lesions (PAI ≥ 3) was 97.7% after 12 months. Of these, 81.8% were classified as “healed,” and 15.9%, as “healing.” Only one tooth was classified as “diseased” [21].

These 3 studies also evaluated postoperative pain associated with GW, using the visual analog scale [21,30,32]. In the first study, which assessed 6-month healing rates, no patient experienced severe pain, while only 3% of the patients experienced moderate pain 2 days after treatment [30]. These observations were updated in the second study, which assessed the 12-month success rate, and in which only 3.8% of the patients experienced moderate pain 2 days after treatment [32]. The third study addressed teeth with a pre-existing periapical lesion (PAI ≥ 3), and indicated that 15.6% of the patients reported mild pain 2 days after treatment. Additionally, at 2, 7, and 14 days after treatment, no patient experienced moderate or severe pain [21]. Another randomized clinical study evaluated the incidence and intensity of postoperative pain using the numeric rating scale for patients who received instrumentation (at least 25.04) plus CSI and PUI (control group), or minimal instrumentation (20.04 or 20.06) plus irrigation with GW (GW group). After 168 hours (7 days), 72% of the patients in the control group had at least one episode of low to mild pain, versus 83.3% of those in the GW group. There was no significant difference between the 2 groups [41].

Although the manufacturer suggests the use of GW in a single-visit [16], different impediments such as time constraints, presence of separated file, device availability make necessary to perform endodontic treatment in multiple visits [21]. The same studies that evaluated postoperative pain, success rate (clinical and radiographic) and healing of periapical lesions also took into consideration the number of visits [21,30,32,41]. After 6 months, a 93.3% success rate was observed in patients who were treated with GW in a single visit. A positive correlation was observed between single-visit and success [30]. However, after 12 months (84.3% recall rate), although the success rate remained high when single-visit was performed, 97.2%, there was no correlation between the number of visits and success [32]. A study that assessed healing of periapical lesions after 12 months (97.7% success rate) revealed that most of the patients (88.9%) were treated in a single visit [21]. Another study that evaluated postoperative pain revealed that 83.3% of patients were treated in 2 visits, with most of the pain eliminated between appointments [41].

10. Limitations

The main limitation of GW is its cost, which would require a financial effort from the clinician. Additionally, GW uses a maximum of 3% NaOCl and 8% EDTA, which can be supplemented with water rinse [32]. This could be considered as a limitation since irrigants at higher concentrations, with different additives (such as surfactants) or alternative/experimental irrigants cannot be used. Regarding the handpiece, it needs vertical space to be attached to the tooth, therefore, structurally compromised teeth should be sufficiently restored to allow a proper attachment, i.e., 1mm above the pulp chamber floor [15]. Another important aspect to be considered is that most of the studies about GW are *in vitro*, and the number of studies that evaluated the topics of the present review was minimal. Additionally, studies that evaluated clinical/radiographic success did not compare GW with other irrigation protocols, nor the association of minimal or conventional instrumentation on success. Thus, more research, especially clinical, is needed to justify the use of GW over other irrigation methods.

CONCLUSIONS

Based on the limited evidence/literature available, the GW System was not associated with extrusion of the irrigant, promoted faster organic dissolution than CSI, PUI CUI and EndoVac, reduced more bacterial DNA and biofilm than PUI and CUI, promoted higher penetration of NaOCl into dentinal tubules than PUI and CUI *in vitro*, and removed more intracanal medication than CSI and PUI. GW was able to remove pulp tissue and calcifications. Moreover, its ability to remove hard-tissue debris and smear layer was better than CSI, and its ability to remove root canal obturation residues was lower or similar to PUI, and similar to CSI and EndoVac. Regarding the root canal obturation of minimally instrumented molar canals, GW was associated with high-quality obturations. Clinically, the success rate of endodontic treatment using GW was 97.3%, and the short-term postoperative pain was no different between the GW and the CSI groups. Further research, mainly clinical, is needed to establish whether GW has any advantages over other available irrigation methods.

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3.4 Publication 4[*]

MULTISPECIES BIOFILM REMOVAL BY A MULTISONIC IRRIGATION SYSTEM IN MANDIBULAR MOLARS

Abstract

Aim To assess biofilm removal efficacy of GentleWave System and passive ultrasonic irrigation (PUI).

Methodology Twenty-two human mandibular molars with Vertucci's type II configuration in the mesial root were selected. Teeth were autoclaved, inoculated with dental plaque and incubated in a CDC biofilm reactor for two weeks. The mesial roots were instrumented up to 20.06 file (V-Taper) for the GentleWave group, and up to 35.04 file (Vortex Blue) for PUI group. Irrigation was performed using GentleWave and PUI irrigation protocols (n=11). Dentine debris on paper points samples were obtained for quantitative real-time Polymerase Chain Reaction (qPCR) and 16S ribosomal RNA gene sequencing (Next Generation Sequencing - NGS). For qPCR, a non-parametric test ($\alpha=0.05$) was used. Next Generation Sequencing data were analyzed using mothur, with alpha diversity calculated as the Shannon and Chao1 indices and Bray-Curtis dissimilarities were used for beta diversity. Differences in alpha diversity and abundances of genera were evaluated using Kruskal-Wallis test. Differences in community composition were evaluated using analysis of similarity with Bonferroni correction for multiple comparisons.

Results qPCR results showed that the reduction estimated in percentages for both groups was equivalent ($P > 0.05$). NGS analysis showed that both techniques promoted a significant reduction of reads and OTUs number ($P < 0.05$). Shannon alpha diversity and Chao1 index showed no differences between pre or post treatment samples for both groups ($P > 0.05$). Additionally, pre-treatment communities differed from post-treatment samples in both groups regarding bacterial taxa reduction (ANOSIM R=0.50 and 0.55, $P < 0.001$).

Conclusions Bacterial reduction in mesial roots of mandibular molars prepared to 35.04 with PUI was similar to those prepared to 20.06 with a multisonic irrigant activation system.

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[*] This is the peer-reviewed version of the article “Multispecies Biofilm Removal by a Multisonic Irrigation System in Mandibular Molars”, which has been published in final form at [https://onlinelibrary.wiley.com/doi/epdf/10.1111/iej.13813] in the *International Endodontic Journal 2022*.

Keywords: biofilms, next-generation sequencing, GentleWave, microbiome, ultrasonic irrigation

Introduction

The main goal of endodontic treatment is to promote the healing of periapical tissues affected by apical periodontitis. To accomplish this goal, the operator needs to eradicate or at least reduce the bacterial concentration in the root canal system (Siqueira & Rôças 2022a,b). However, disinfection may be challenging when bacteria are organized in multispecies matrix-enclosed communities called biofilms, especially in teeth with complex anatomies. These bacterial structures can colonize the canal walls (Manoharan et al., 2020), ramifications and isthmuses (Ricucci & Siqueira 2010).

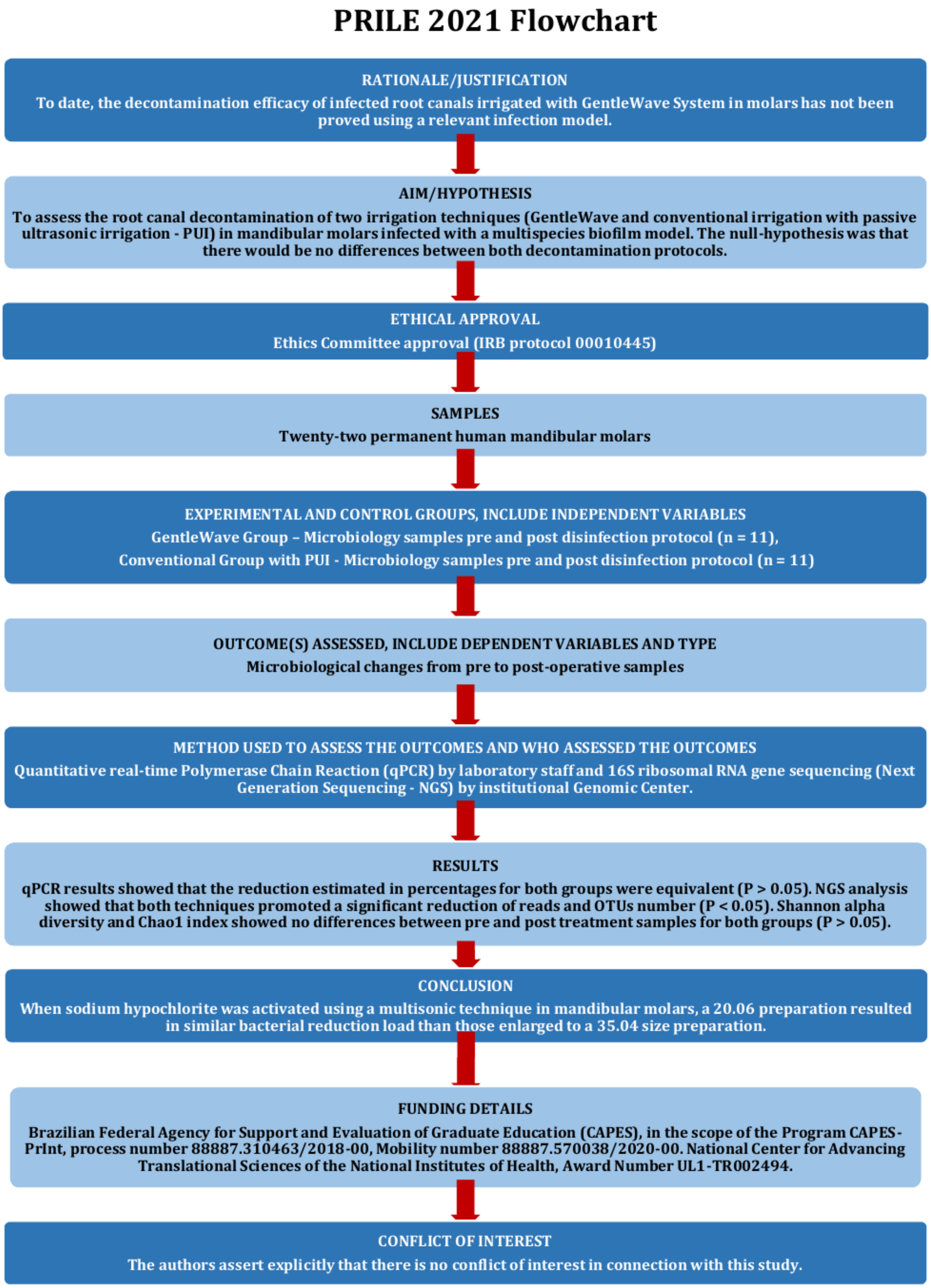
Traditionally, culture methods have been used to assess the bacterial composition and decontamination of the root canal system (Siqueira & Rôças 2022a). This method allows a semi- or absolute quantification of culturable bacteria. However, a significant amount of microorganisms in the root canal space cannot be cultured under laboratory conditions. The development of The Human Genome Project (Venter et al., 2001) allowed the subsequent development of databases (i.e., SILVA) for use in conjunction with next generation sequencing (NGS) technologies (van Dijk et al., 2018; Zhong et al., 2021). Next generation sequencing is a fifth-generation laboratory tool of microbiological analysis for the study of endodontic infections. This method provides vast information about bacterial communities and their profiles (Manoil et al., 2020; Siqueira & Rôças 2022a).

The introduction of NGS brought new knowledge related to the composition of secondary endodontic infections. During the last 2 decades culture and closed-end methods such as PCR showed that *Enterococcus faecalis* was the most common bacteria associated with secondary or persistent endodontic infections (Sundqvist et al., 1998; Molander et al., 1998; Pinheiro et al., 2003; Rôças et al., 2004; Siqueira & Rôças 2004). However, this knowledge has been challenged by recent studies that revealed a complex bacterial composition present in failed cases (Anderson et al., 2013; Tzanetakis et al., 2015; Siqueira et al., 2016; Keskin et al., 2017; Bouillaguet et al., 2018; Sánchez-Sanhueza et al., 2018). Given the complex microbial composition of secondary root canal infections, it is necessary to develop novel tooth models inoculated with multispecies biofilm, in order to challenge endodontic disinfection procedures.

Recently, a new system that combines multisonic and negative apical pressure (GentleWave, Sonendo Inc, Laguna Hills, CA, USA) was introduced for cleaning and disinfecting root canals (Haapasalo et al., 2014) using minimal preparation sizes, i.e., using small files with sizes #15 or #20 tip (Coaguila-Llerena et al., 2022). This system creates hydrodynamic cavitation in the root canal space, and the implosion of microbubbles creates an acoustic field of broadband frequencies that travels through the fluid to the entire root canal system (Sigurdsson et al., 2018). To date, the decontamination efficacy of infected root canals irrigated with this method in molars has not been proved using a relevant infection model. This study aimed to assess the root canal decontamination of two irrigation techniques (GentleWave and passive ultrasonic irrigation - PUI) in mandibular molars infected with a multispecies biofilm model. The null-hypothesis was that there would be no differences between both decontamination protocols.

Materials and Methods (extended methodology in APPENDIX C)

This study is reported in accordance with the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021). The PRILE 2021 flowchart is presented in Fig. 1.



PRILE 2021 Flowchart

RATIONALE/JUSTIFICATION

To date, the decontamination efficacy of infected root canals irrigated with GentleWave System in molars has not been proved using a relevant infection model.

AIM/HYPOTHESIS

To assess the root canal decontamination of two irrigation techniques (GentleWave and conventional irrigation with passive ultrasonic irrigation - PUI) in mandibular molars infected with a multispecies biofilm model. The null-hypothesis was that there would be no differences between both decontamination protocols.

ETHICAL APPROVAL

Ethics Committee approval (IRB protocol 00010445)

SAMPLES

Twenty-two permanent human mandibular molars

EXPERIMENTAL AND CONTROL GROUPS, INCLUDE INDEPENDENT VARIABLES

GentleWave Group – Microbiology samples pre and post disinfection protocol (n = 11),

Conventional Group with PUI - Microbiology samples pre and post disinfection protocol (n = 11)

OUTCOME(S) ASSESSED, INCLUDE DEPENDENT VARIABLES AND TYPE

Microbiological changes from pre to post-operative samples

METHOD USED TO ASSESS THE OUTCOMES AND WHO ASSESSED THE OUTCOMES

Quantitative real-time Polymerase Chain Reaction (qPCR) by laboratory staff and 16S ribosomal RNA gene sequencing (Next Generation Sequencing - NGS) by institutional Genomic Center.

RESULTS

qPCR results showed that the reduction estimated in percentages for both groups were equivalent ($P > 0.05$). NGS analysis showed that both techniques promoted a significant reduction of reads and OTUs number ($P < 0.05$). Shannon alpha diversity and Chao1 index showed no differences between pre and post treatment samples for both groups ($P > 0.05$).

CONCLUSION

When sodium hypochlorite was activated using a multisonic technique in mandibular molars, a 20.06 preparation resulted in similar bacterial reduction load than those enlarged to a 35.04 size preparation.

FUNDING DETAILS

Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), in the scope of the Program CAPES-PrInt, process number 88887.310463/2018-00, Mobility number 88887.570038/2020-00. National Center for Advancing Translational Sciences of the National Institutes of Health, Award Number UL1-TR002494.

CONFLICT OF INTEREST

The authors assert explicitly that there is no conflict of interest in connection with this study.

Figure 1 *From: Nagendrababu V, Murray PE, Ordinola-Zapata R, Peters OA, Rôças IN, Siqueira JF Jr, Priya E, Jayaraman J, Pulikkotil SJ, Camilleri J, Boutsioukis C, Rossi-Fedele G, Dummer PMH (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontology: a consensus-based development. International Endodontic Journal. doi: 10.1111/iej.13542. https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13542.
For further details visit: http://pride-endodonticguidelines.org/prile/

Specimen selection

A power calculation was performed in a previous study to determine the sample size necessary (n=11) to distinguish differences between pre- and post-treatment communities (taken as observed abundance of genera) using the HMP package in R software (la Rosa et al., 2012; Ordinola-Zapata et al., 2022). After Ethics Committee approval (CAAE: 37090820.3.0000.5416) (ATTACHMENT B), 22 permanent human mandibular molars, extracted for reasons not related to this study, were used. Teeth were selected following these inclusion criteria: intact apices, no extensive restorations, absence of calcifications, root canal curvature between 20 and 40°, and type II Vertucci's configuration in mesial roots confirmed by Micro-CT scanning (Skyscan 1176, Bruker-MicroCT, Kontich, Belgium). After the access cavity and patency, the working length (1-mm short of apical foramen) were confirmed by using an ISO size 10 K-file (Dentsply Sirona, York, PA, USA). Afterwards, teeth were autoclaved and stored in distilled water.

Root canal contamination

The multispecies biofilm was obtained by using a human subgingival dental plaque sample from a single healthy donor (BPE 0-1), which was obtained from interdental spaces of mandibular molars using sterile inoculating needles (Fisher scientific, Hampton, NH, USA), as previously reported (Ordinola-Zapata et al., 2022). The sample was placed and diluted in anaerobic transport medium (Anaerobe System, Morgan Hills, CA, USA). The root canals were coated first with 0.1mL of sheep blood (HemoStat Laboratories, Dixon, CA, USA) to promote the formation of an organic layer, and then 100µL of diluted dental plaque was introduced. The teeth were mounted on a custom-built stand. This stand was placed in the Center for Disease Control (CDC) reactor (BioSurface Technologies, Bozeman, MT, USA), which produces conditions for an oral microcosm model (Fig. 2). All the reactor components were autoclaved. The reactor has a lidded vessel that allows a defined flow of the 350mL Columbia medium (Difco, BD, Sparks, MD, USA) and has a stir bar that generates shear forces (Rudney et al., 2012). The internal temperature of the CDC vessel was set at 37°C and 90 rpm stirring rate. Initially, the reactor was incubated under shear conditions, without media flow, for 24h; then, Columbia medium was pumped into the reactor at 2.5 L/24 h flow rate for 2 weeks. The presence of an amorphous biofilm layer covering diverse substrates including the root canal wall surface has been previously validated (Rudney et al., 2012; Li et al., 2014; Ordinola-Zapata et al., 2022). The teeth were then removed for treatment.

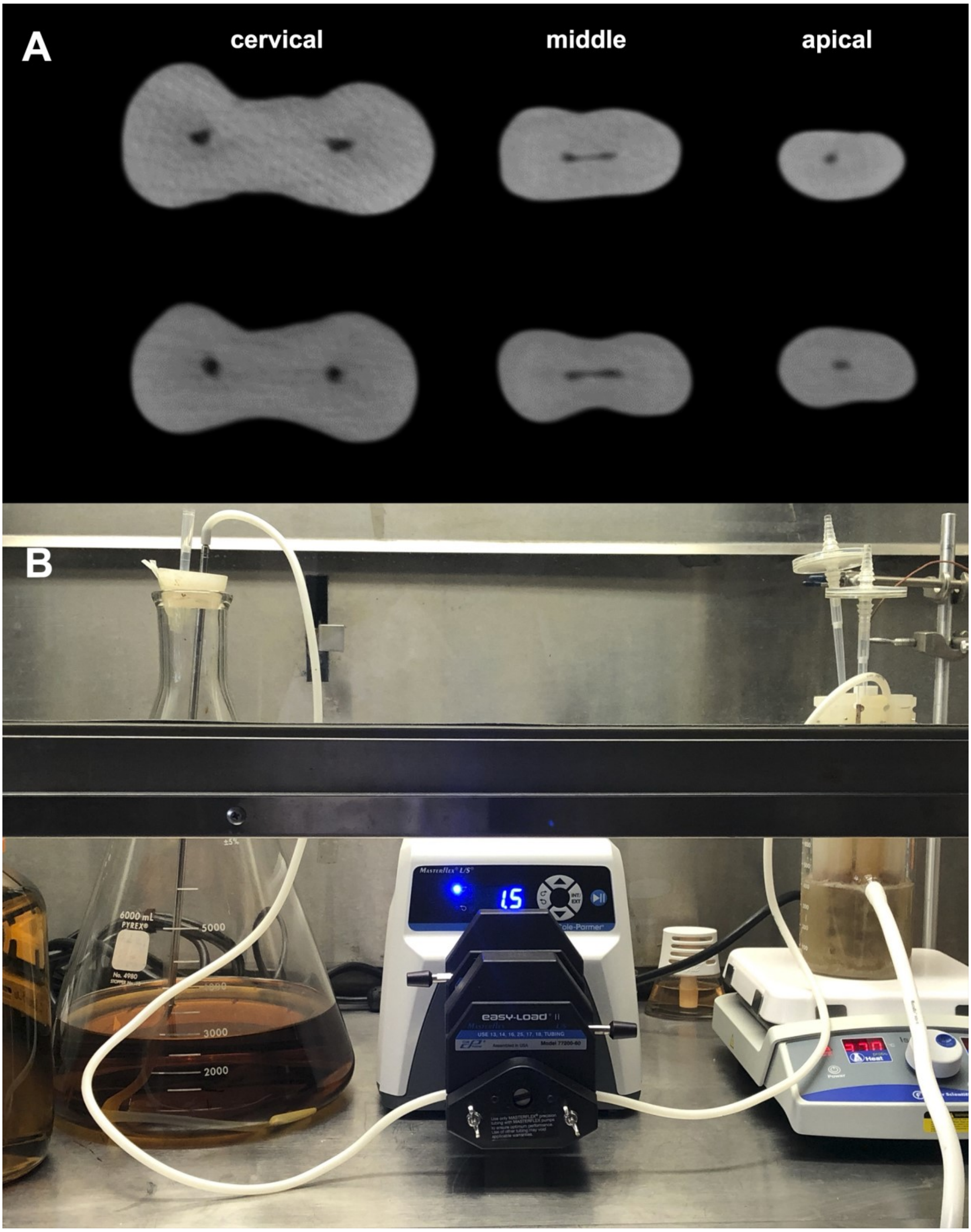


Figure 2 A) Representative cross-sectional micro–CT images of two samples scanned at 35μm voxel size, showing a type **II** Vertucci's configuration in the mesial roots. B) Center for Disease Control (CDC) reactor (BioSurface Technologies, Bozeman, MT, USA) used in the present study.

Root canal preparation, final irrigation protocols and microbial sampling

The external surface of the crowns of accessed teeth were decontaminated with sodium hypochlorite (NaOCl) at 6% (Clorox, Oakland, CA, USA) for 3 min and then with 10% sodium thiosulfate for 3 min. The decontamination protocol was previously verified in a pilot study confirming removal of bacterial DNA (data not shown). In the sequence, the mesial canals were instrumented with ISO size 15 Hedstrom files (Dentsply Sirona) using a filling motion for 30s to generate dentine shavings. Then, sterile paper points were placed to absorb the root canal content with shavings (pre-operative sample). The teeth were randomly distributed into two groups: GentleWave (n=11) and PUI (n=11). For the GentleWave group, a minimal instrumentation was performed with a 20.06 variable taper file (V-Taper, SSWhite, Lakewood, NJ, USA) following manufacturer recommendations. For PUI group, conventional instrumentation up to 35.04 file (Vortex Blue, Dentsply) was performed. All canals were irrigated with 10mL of 3% NaOCl (Clorox, diluted and titrated from a 6% NaOCl solution) during the instrumentation process using a 3-mL syringe and a 30G side-vented needle 2-3mm short of working length. All apices were sealed with cyanoacrylate and sterile red wax to obtain a closed system for irrigation. The agitation protocols were:

PUI group: for final irrigation, three percent NaOCl was activated in each canal (MB and ML), 2-mm short of the working length, using a 20.02 ultrasonic tip coupled to a piezoelectric device (EndoUltra, Vista Dental, Racine, WI, USA) at 40,000 Hz frequency. Two mL of the solution was activated for 20s, the procedure was repeated three times for a total final irrigation time of 1 minute. The total NaOCl volume of final irrigation per canal was 6mL. The same procedure was repeated for 17% EDTA.

GentleWave Group: The access of all teeth was sealed using a barrier (Soundseal, Sonendo). The pulp chamber floor was gauged to have the procedure instrument tip 1-2mm above the pulp floor. The irrigation protocol consisted in 1-min cycle with distilled water, 4-min cycle with 3% NaOCl, 1- min cycle with 8.5% EDTA and finally 1-min cycle with distilled water.

After final irrigation, all canals were irrigated with 2mL of 10% sodium thiosulfate for 3min to inactive the NaOCl carry-over effect. Postoperative microbiological samples from both groups were obtained using ISO size 15 Hedstroem files in a filing motion for 30 seconds (Dentsply Sirona). Sterile paper points were used to obtain the samples (post-operative sample). The pre- and post-operative samples (paper points) were placed in anaerobic transport medium (Anaerobe System, Morgan Hills, CA, USA) and vortexed for 10s.

Quantitative real-time PCR analysis (qPCR), DNA extraction and sequencing analysis

The DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) was used to extract the DNA. The institution Genomic Center processed the samples for qPCR quantification and results were expressed in molecules/µL. For DNA sequencing, the V3-V4 hypervariable region of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced using paired-end sequencing at 301 nucleotides (nt) read length on the Illumina MiSeq platform by the dual-index method. Raw data returned as ".fastq" files and uploaded in the Sequence Read Archive under BioProject accession number SRP328673.

Amplicon Processing and Analysis

Sequence data were processed and analysed using mothur ver. 1.41.1 (Schloss 2020). Sequences were first trimmed to the first 250 nt and paired-end joined using fastq-join software. Quality trimming was performed at a threshold of 35 over a sliding window of 50 nt. In addition, sequences with homopolymers >8 nt, ambiguous bases, or >2 mismatches from primer sequences were removed. High-quality sequences were aligned against SILVA database ver. 138.1 for downstream processing. Chimeras were identified and removed using UCHIME ver. 4.2.40. Sequencing errors were further removed using a 2% pre-clustering step. Operational taxonomic units (OTUs) were binned at a similarity of 99% using the furthest-neighbour algorithm and were classified against the version 18 release from the Ribosomal Database Project (Cole et al., 2009). Different databases were used for alignment and classification due to processing considerations described previously (Schloss & Westcott 2011).

Statistical analysis

For qPCR, data were transformed to Log values and the percentage of reduction between pre- and post-operative samples was analysed using Mann-Whitney U test (GraphPad Software, La Jolla, CA, USA). For microbiome analysis, Kruskal-Wallis test was used to evaluate differences in alpha diversity and abundances of genera, with Dunn's *post-hoc* test and Bonferroni correction for multiple comparisons using, XLSTAT ver. 2020.2.3 (Addinsoft; Belmont, MA, USA). The Shannon and Chao1 indices were used to measure alpha (within-sample) diversity. Paired analyses were used when indicated. The Bray-Curtis dissimilarity was used to measure beta (between-groups) diversity and was visualized by ordination using principal coordinate analysis. Differences in community composition were evaluated using analysis of similarity (ANOSIM), with Bonferroni correction for multiple comparisons (Clarke 1993).

Results

The analysis with qPCR showed that the PUI group promoted a reduction from 5.95 molecules/µL $\log_{10}$ (pre-operative) to 1.75 qPCR molecules/µL $\log_{10}$ (post-operative), while GentleWave had 5.6 molecules/µL $\log_{10}$ (pre-operative) and 0.44 molecules/µL $\log_{10}$ (post-operative). The reduction estimated in percentages indicates that both groups were equivalent ($P > 0.05$) (Table 1). Multiple diversity assessments revealed that preoperative samples in both groups had no significant difference in the bacterial composition. NGS analysis showed that fewer reads and OTUs were obtained from post-operative samples in both experimental groups ($P < 0.0001$). Shannon alpha diversity and Chao1 indexes showed no differences for both groups ($P > 0.05$) (Table 2). Both groups promoted significant reduction in relative abundances of *Parvimonas* and *Prevotella*. The PUI group promoted significant reduction in relative abundance of *Fusobacterium* (Fig. 3). Beta diversity analysis showed no differences in pre or post treatment communities in both groups (ANOSIM R=0.08 and 0.02; $P = 0.088$ and 0.025, respectively; Bonferroni-corrected $\alpha = 0.008$). Additionally, pre-treatment communities differed from post-treatment samples in both groups regarding bacterial taxa reduction (ANOSIM R=0.50 and 0.55, $P < 0.001$) (Fig. 3).

Groups	Pre	Post	Reduction (%)
PUI	5.95 (0.68)	1.75 (0.96)	78.5 (51.8 - 100) ^{A}
GW	5.60 (0.83)	0.44 (0.70)	100 (66.9 - 100) ^{A}

Table 1. qPCR molecules/µL $\log_{10}$ of multispecies biofilm before and after treatment with PUI or GentleWave treatment.
Values are expressed as mean (SD). Percentage reduction is expressed in terms of median and range. Similar uppercase letter in the column represents no significant differences between groups.

Treatment	Time	Reads	Coverage (%)	OTUs	Shannon	Chao 1
PUI	Pre	85,711 ± 23,230 ^{A}	100 ± 0.00	631 ± 202 ^{A}	3.15 ± 0.13 ^{A}	1974 ± 743 ^{A}
	Post	19,068 ± 33,679 ^{BC}	99 ± 0.02	51 ± 37 ^{B}	2.37 ± 0.64 ^{BC}	63 ± 55 ^{B}
GW	Pre	58,016 ± 27,957 ^{AB}	99 ± 0.00	439 ± 191 ^{A}	2.98 ± 0.24 ^{AB}	1297 ± 630 ^{A}
	Post	4,632 ± 13,247 ^{C}	87 ± 0.30*	27 ± 27 ^{B}	2.20 ± 0.68 ^{C}	40 ± 60 ^{B}
	P value	< .0001	.344	< .0001	< .0001	< .0001

Table 2. Mean and standard deviation of sequencing data from all samples, including Shannon and Chao1 alpha diversity indices. Different uppercase letters in each column indicate significant differences by Dunn's *post-hoc* test (*P* < .05). PUI: passive ultrasonic irrigation; GW: GentleWave System; OTUs: operational taxonomic units.

* 2 post-treatment samples had negligible amount of DNA and reads; coverage was 0 and 0.71.

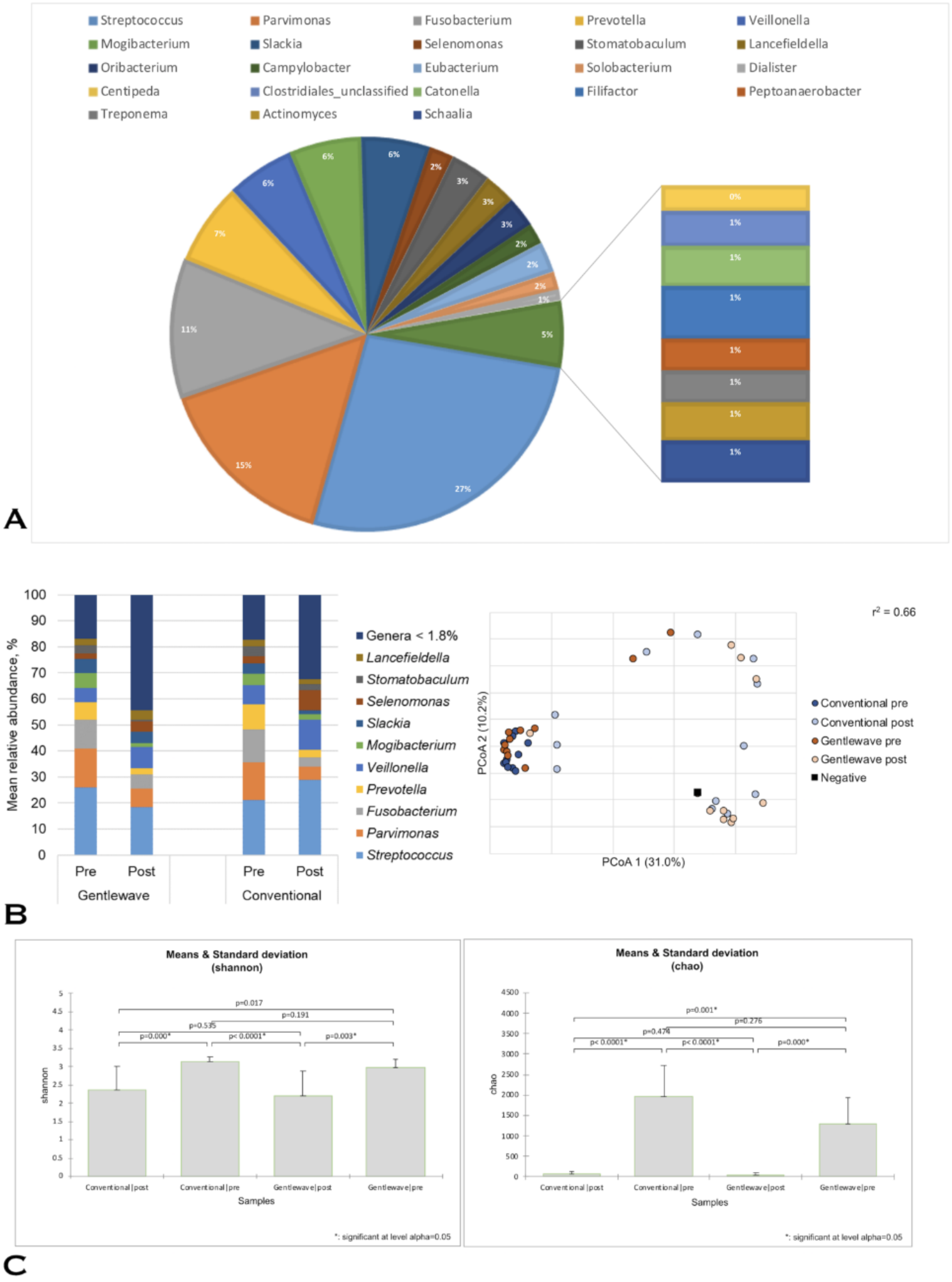


Figure 3 A) Composition of microbial communities associated with the root canal disinfection model used in this study (preoperative samples). B) Distribution of abundant genera among groups. Genera with a mean abundance < 1.8% among all samples were consolidated. Principal coordinate analysis of Bray-Curtis dissimilarity matrices (beta diversity). C) Shannon and Chao1 alpha diversity. Conventional=PUI.

Discussion

The present study assessed the multispecies biofilm removal ability of GentleWave and PUI in mesial roots of human mandibular molars. The null-hypothesis was accepted because no differences were found between both treatment strategies. The 16S rRNA gene presents a combination of conserved, variable and hypervariable regions, the latter of which have made it the most sequenced taxonomic marker for the characterization of microbial community diversity (D'Amore et al., 2016). In the present study, the biofilm removal was assessed by 16S rRNA amplicon sequencing using NGS technology, which is an open-ended analysis. This allows the detection of the vast majority and most dominant bacteria in a root canal sample (Siqueira & Rôças 2022a). Thus, it has become a standard method in basic biology (van Dijk et al., 2018; Siqueira & Rôças 2022a).

The production of multispecies biofilm was performed using the CDC reactor. Although biofilm composition is different for each individual, the reactor generates reproducible microcosm biofilms closely representative of the oral microbiota (Rudney et al., 2012; Li et al., 2014). The microbial community retains more than 60% of the inoculated species under a controllable homogeneous environment (Rudney et al., 2012). Additionally, specimens can be incubated at predetermined times for biofilm assessment (Li et al., 2014). It is important to consider that an "old" biofilm (weeks) differs from a "young" one (days) in terms of biomass/thickness, cell count and antimicrobial resistance (Swimberghe et al., 2019). In the present study, the incubation time was 2 weeks because it has been shown that a polymicrobial biofilm reaches its maturation at this timeframe (Stojicic et al., 2013). In this study, the biofilm was dominated by 10 bacterial taxa including *Streptococcus, Parvimonas, Fusobacterium, Prevotella, Veillonella, Mogibacterium, Slackia, Selenomonas, Stomatobaculum* and *Lancefieldella* which represented a significant proportion of the microbial population. These species have been found consistently in cases of primary and secondary endodontic infections (Manoharan et al., 2020; Manoil et al., 2020). More specifically, a study proposed that the genera *Streptococcus, Prevotella, Parvimonas, Fusobacterium,* and *Veillonella* are non-motile bacteria that play an important role in microbiome cargo-transport which shaped the spatial organization of a microbial community (Shrivastava et al., 2018). Similar to a previous study (Ordinola-Zapata et al., 2022), the *Streptococcus* and *Veillonella* taxa persisted in post-operative samples, regardless of the disinfection protocol. This can be explained by the high concentration of these bacteria before treatment, and the adhesion of these bacteria to the dentinal substrate (Love & Jenkinson 2002; Do et al., 2015), which would allow deep colonization of the dentinal tubules, and therefore making their removal difficult.

Multispecies biofilm removal was assessed in human molars considering two different instrumentation approaches: large and minimal apical size instrumentation. For PUI group, instrumentation was performed up to 35.04 instrument because it promotes a significant reduction in endotoxin levels (Marinho et al., 2012) and to allow free placement of the irrigation tip in the canal (Van Der Sluis et al., 2007). For GentleWave, a minimal instrumentation was performed as recommended by the manufacturer (GentleWave Datasheet). In the present study, the 20.06 file was used for GentleWave as previously reported (Grigsby et al., 2020). Although the tip size is small, the taper is .06, which may favour the irrigant flow (Boutsioukis et al., 2010). It is important to note that the standardization of both groups in anatomic and microbiological terms did not remove the intrinsic differences in the irrigation protocols assessed. It was not possible to match the irrigation protocols for PUI and GentleWave groups in terms of canal size, taper, and irrigant volume. Thus, results must be interpreted carefully. GentleWave uses approximately 45mL/min flow rate (Haapasalo et al., 2014), being 180mL of NaOCl, and approximately 270mL total volume of irrigant solutions, whereas 10mL of needle irrigation plus 6 mL of ultrasonic irrigation per canal were used in the PUI group. Trying to match the irrigation protocols is impractical, because the PUI irrigation may need approximately more than 10-20X of continuous activation at a rate of 6mL per minute in each canal to match at least the NaOCl volume. This increase in activation time increases the chances of ultrasonic tip separation and other procedural errors (i.e., ledge, and uncontrolled dentine removal) (Retsas et al., 2016). In addition, a 30 min ultrasonic activation per canal might be considered unrealistic and impractical for clinical use. The instrumentation of the canals was also not standardized, being 20.06 for GentleWave group and 35.04 for PUI group. In this regard, #15 (Zhang et al., 2019) or #20 (Sigurdsson et al., 2018) apical sizes have been used as minimal instrumentation sizes in previous studies. The gene sequencing analysis showed that there was no difference between GentleWave and PUI group, regardless of the instrumentation size. Furthermore, a previous study showed that there was no difference between the use of GentleWave using minimal (15.04) and conventional (35.04) instrumentation in the reduction of *E. faecalis* lipoteichoic acid (Velardi et al., 2022). In this regard, a study revealed that the apical diameter is not a relevant factor when the irrigant is activated (Lee et al., 2019). Based on our results, the instrumentation up to 20.06 in mesial roots of mandibular molars in combination with a multisonic irrigation protocol could be advantageous considering the reduced amount of pericervical dentine removal and the decreased chances to create instrumentation error procedures.

Some limitations were observed in the biofilm model used in this research. The magnitude of reads and OTUs per sample is significantly higher than the amount of OTUs found in clinical cases by a magnitude of 20-100X (Manoil et al., 2020). This shows that the *in-vitro* model could be more challenging to disinfect than an infected tooth with a necrotic pulp. On the other hand, the model allowed us to obtain a standardized microcosm that is representative of the primary root canal infection. Another limitation is that the results should be considered as a surrogate, because the success of endodontic treatment not only relies on the antimicrobial effect of the treatment but also on pre-operative and post-treatment clinical factors. Thus, this model simulation cannot unambiguously establish causality between success or treatment failure.

Conclusion

Bacterial reduction in mesial roots of mandibular molars prepared to 35.04 with PUI was similar to those prepared to 20.06 with a multisonic irrigant activation system.

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4 CONCLUSIONS

Part 1:

-The addition of surfactants to $Ca(OCl)_2$ did not increase the penetration into dentinal tubules, but it did promote lower surface tension, without changing the pH or free available chlorine values, and higher availability of free calcium ions in $Ca(OCl)_2$ (Publication 1).

-Although $Ca(OCl)_2$ and NaOCl promoted the same cytotoxicity mechanism and did not stimulate ALP activity, $Ca(OCl)_2$ was less cytotoxic than NaOCl (Publication 2).

Part 2:

-Further research, mainly clinical, is needed to establish whether GentleWave has any advantages over other available irrigation methods (Publication 3).

-There was no difference between GentleWave and Passive Ultrasonic Irrigation (PUI) in the reduction of multispecies biofilm; bacterial reduction in mesial roots of mandibular molars prepared to 35.04 with PUI was similar to those prepared to 20.06 with a GentleWave (Publication 4).

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APPENDIX A – EXTENDED METHODOLOGY (IN PORTUGUESE) OF PUBLICATION 1

“**Physicochemical properties and penetration into dentinal tubules of calcium hypochlorite with surfactants**”

Preparo das soluções irrigantes e determinação de tensão superficial e CMC

As soluções estoque de $Ca(OCl)_2$ e NaOCl foram preparadas a aproximadamente 6% peso/volume (p/v). O $Ca(OCl)_2$ foi preparado diluindo o hipoclorito de cálcio em pó (Êxodo Científica, Sumaré, SP, Brasil) em água destilada sob agitação constante por 30 minutos e, em seguida, filtrando a solução com papel de filtro para remover o sedimento. O NaOCl foi preparado diluindo NaOCl a 10% (AraQuímica, Araraquara, SP, Brasil) em água destilada. Em seguida, o cloro livre de ambas as soluções estoque foi determinado pelo método de titulação de iodo/tiossulfato de sódio. As soluções foram armazenadas em geladeira por no máximo 20 dias até o uso, a 4°C, ao abrigo da luz. Para a avaliação das propriedades físico-químicas, as soluções foram retiradas do refrigerador e mantidas na sala até que a temperatura das soluções se igualasse à temperatura ambiente determinada para cada ensaio.

$Ca(OCl)_2$ a 2,5% e NaOCl a 2,5%, com ou sem cloreto de benzalcônio, cetrimida, Tween 80 e Triton X-100 (Sigma-Aldrich, St. Louis, MO, EUA), foram preparados a partir de soluções estoque em diferentes concentrações (Tabela 1).

A tensão superficial de todas as misturas (n = 3) foi medida usando o método de gota pendente em temperatura ambiente de 22°-24°C. Cada solução foi colocada em uma seringa acoplada a um sistema OCA-20 (DataPhysics Instruments, Filderstadt, Alemanha), onde formou uma gota que capturada digitalmente por um dispositivo de carga acoplada. A tensão superficial foi calculada automaticamente com base na forma da gota, usando um programa de software SCA-20 (DataPhysics Instruments), conforme descrito anteriormente (9). Em seguida, os dados de tensão superficial foram plotados em um programa de software Origin 8 (OriginLab, Northampton, MA, EUA), e uma regressão linear da curva foi aplicada nos eixos “x” (concentração de surfactante) e “y” (tensão superficial). A intersecção das duas linhas (resultantes dos eixos “x” e “y”) permitiu calcular a CMC e determinar a tensão superficial na CMC. O NaOCl a 2,5% e o $Ca(OCl)_2$ a 2,5%, misturados com os surfactantes na CMC, foram usados para avaliar o pH, cloro livre e íons cálcio livres.

Determinação do pH

NaOCl a 2,5% e $Ca(OCl)_2$ a 2,5% recém-preparados, com e sem surfactantes na CMC (n = 3), foram agitados. Em seguida, o pH de cada solução foi medido com um pHmetro (DM-22, Digimed, São Paulo, SP, Brasil) à temperatura ambiente de 22°C, de acordo com os requisitos da Farmacopeia Europeia.

Determinação do cloro livre

O cloro livre foi determinado pelo método de titulação de iodo/tiossulfato de sódio (n = 3). Um total de 10 mL de NaOCl a 2,5% diluído e $Ca(OCl)_2$ a 2,5% diluído (5g cada em 100 mL de água destilada), com ou sem surfactantes na CMC, foi misturado com 30 mL de iodeto de potássio 5% (Êxodo Científica), e com 10 mL de ácido acético 99,8% (Neon Comercial, Suzano, SP, Brasil). Em seguida, uma solução de tiossulfato de sódio a 0,1 N previamente padronizada (Labsynth Produtos para Laboratórios, Diadema, SP, Brasil) foi gotejada no NaOCl e $Ca(OCl)_2$ utilizando uma bureta padrão de 50 mL, até que se tornassem amarelo pálido. Imediatamente, foi adicionada uma solução de amido a 0,5% (Êxodo Científica), tornando a solução de coloração azul intensa. Subsequentemente, a solução de tiossulfato de sódio a 0,1 N

(Labsynth) foi gotejada na solução azul até ficar transparente. O volume necessário de tiossulfato de sódio foi registrado (10). A temperatura ambiente foi mantida a 20°C (14). Os dados foram expressos em porcentagem (%) de p/v.

Determinação de íons de cálcio livre

A concentração de íons de cálcio livre no $Ca(OCl)_2$ a 2,5% com ou sem surfactantes na CMC (n = 3) foi determinada à temperatura ambiente de 22°-24°C por potenciometria, usando um eletrodo seletivo de cálcio. O potenciômetro (Bante Instruments, Sugar Land, TX, EUA) permitiu medir a condutividade de íons de cálcio livre (expressa em concentração mmol/L) usando uma curva de calibração (R^2=0,9996) a partir de uma solução padrão de cálcio (0,1 M). Em seguida, 10 µL a 40 µL das soluções foram adicionados sequencialmente em 20 mL de água destilada, e um eletrodo foi colocado a cada adição para obter as medidas. Os íons de cálcio livre esperado foi calculado a cada adição de 20 mL de água destilada mais 10 µL a 40 µL das soluções experimentais. Este cálculo teórico foi realizado considerando que a fórmula para $Ca(OCl)_2$ tem 2 mols de íon hipoclorito para 1 mol de íon cálcio.

Penetração nos túbulos dentinários

Este ensaio foi realizado selecionando os surfactantes cloreto de benzalcônio e Triton X-100, que promoveram a menor tensão superficial tanto em NaOCl a 2,5% quanto $Ca(OCl)_2$ a 2,5%. O tamanho da amostra foi calculado usando o programa de software G* Power 3.1.7 para Windows (Heinrich-Heine-Universität Düsseldorf, Alemanha). O cálculo foi baseado em um tamanho de efeito = 0,44 (com base em um estudo piloto), poder de teste (β) = 0,8 e α = 0,05, usando a família de teste F para análise unidirecional, e mostrou que 72 espécimes (n = 12) eram necessários.

Após a aprovação do estudo pelo Comitê de Ética da Faculdade de Odontologia (CAAE: 09799019.4.0000.5416) [**ATTACHMENT A**], 75 pré-molares humanos, permanentes, unirradiculares, recém-extraídos, doados pelo banco de dentes, foram limpados de quaisquer marcas de tecidos residuais, desinfetados em 2,5%. NaOCl por 5 min, e armazenado em timol 0,1% a 4°C até o uso. Os critérios de exclusão foram: dentes com mais de um canal radicular/forame apical, canais ovais, tratamento prévio, calcificação, reabsorção interna/externa, trincas, fraturas na superfície radicular, ângulo de Schneider > 5° ou canais que permitissem a inserção de uma lima que exceda uma lima tipo K15 no forame apical. Para confirmar os critérios de inclusão, os dentes foram examinados em estereomicroscópio (Leica Microsystems, Wetzlar, Alemanha) e radiografados com um sensor digital (FONA CDR Elite, Schick by Sirona Dental, Long Island, NY, EUA). A análise radiográfica foi realizada a partir das projeções mésio-distal e vestíbulo-lingual, para selecionar os dentes com dimensões semelhantes, canais únicos, arredondados, raízes retas (ângulo de Schneider < 5°) e forame único (configuração tipo I de Vertucci). As imagens radiográficas foram analisadas usando um programa de software Image J (National Institutes of Health, Bethesda, MD, EUA). Os canais radiculares foram considerados arredondados quando o diâmetro vestíbulo-lingual se igualava ao diâmetro mesiodistal. Após a seleção, os espécimes foram alocados aleatoriamente em seis grupos experimentais (n = 12) usando um algoritmo de computador (http://www.random.org) para garantir uma distribuição homogênea. As coroas foram removidas e o comprimento da raiz foi padronizado em 16 mm. Os canais radiculares foram instrumentados com o sistema ProDesign Logic (Easy Equipamentos Odontologicos, Belo Horizonte, MG, Brasil). Com 15 mm de comprimento de trabalho, as limas 25,01, 25,03, 25,05 e 40,05 foram utilizadas na velocidade de 350-600 rpm e torque de 1-4 Ncm, dependendo da lima, usando um motor elétrico (VDW Silver, VDW, Munique, Alemanha) (9). Os canais radiculares foram irrigados com 2 mL de NaOCl a 2,5% por 1 minuto a cada troca de lima, seguido de irrigação com 5 mL de EDTA a 17% por 3 minutos e 5 mL de água destilada. Os canais foram secos com cones de papel equivalentes à última lima. Em seguida, os canais foram preenchidos com solução de cristal violeta a 1% (Labsynth Produtos para Laboratórios, Diadema, SP, Brasil), e mantidos a 37°C e 95% de umidade relativa por 3 dias. Em seguida, foram irrigados com 20 mL de água destilada e o ápice foi selado com resina composta para criar um sistema fechado (1). Os grupos experimentais foram distribuídos da seguinte forma: $Ca(OCl)_2$ a 2,5% + cloreto de benzalcônio, $Ca(OCl)_2$ a 2,5% + Triton X-100, NaOCl a 2,5% + cloreto de benzalcônio, NaOCl a 2,5% + Triton X-100, $Ca(OCl)_2$ a 2,5% e NaOCl a 2,5%. É importante salientar que os grupos com os surfactantes cloreto de benzalcônio e Triton X-100 se utilizou a CMC previamente determinada. Três dentes foram irrigados com água destilada para servir como controles da reação. Em seguida, os espécimes foram irrigados em temperatura ambiente de 22-24°C com 5 mL das soluções irrigadoras por 2 minutos (1) usando uma seringa de 5 mL (Ultradent Products, South Jordan, UT, EUA) acoplada a uma agulha de saída lateral 27G (Endo-Eze®, Ultradent Products), posicionada 2mm aquém do comprimento de trabalho. Os canais radiculares de todos os grupos foram irrigados com 5 mL de água destilada e seccionados transversalmente ao longo de seu eixo longitudinal a 3, 7 e 12 mm do ápice, utilizando uma máquina de corte de baixa velocidade (Isomet 1000, São Paulo, SP, Brasil), para obter segmentos dos segmentos cervical, médio e apical. A superfície cervical de cada segmento foi polida com lixa de grão 1000 (3M ESPE, St. Paul, MN, EUA), sob irrigação constante com água. Um estereomicroscópio (LeicaM80, Leica Microsystems) e o software Leica Application Suite EZ 3.0 (Leica Microsystems) foram usados para obter as imagens. A profundidade de penetração foi medida em micrômetros (μm) em 10 regiões equidistantes usando o programa Image J (National Institutes of Health, NIH) (9). Um examinador previamente calibrado e às cegas realizou as medidas duas vezes, com intervalo de 2 semanas (coeficiente de correlação intraclasse > 0,9).

APPENDIX B – EXTENDED METHODOLOGY (IN PORTUGUESE) OF PUBLICATION 2

“Calcium hypochlorite cytotoxicity mechanism in fibroblasts and effect on osteoblast biology”

Preparo das soluções irrigantes

As soluções estoque de $Ca(OCl)_2$ e NaOCl foram preparadas a aproximadamente 6%. O $Ca(OCl)_2$ foi preparado diluindo o pó de $Ca(OCl)_2$ (Êxodo Científica, Sumaré, SP, Brasil) em água destilada (aproximadamente 6% p/v). A solução resultante foi agitada por 30 minutos e, em seguida, filtrada com papel de filtro. O NaOCl foi preparado diluindo uma solução de NaOCl a 10% (AraQuímica, Araraquara, SP, Brasil) em água destilada. Ambas as soluções estoque foram tituladas usando o método de titulação de iodo/tiossulfato de sódio (Vogel 1962) para determinar o cloro livre e foram armazenadas a 4°C por não mais que 20 dias, ao abrigo da luz. As soluções de $Ca(OCl)_2$ a 2,5% e NaOCl a 2,5% foram preparadas diluindo as soluções estoque tituladas e submetendo-as a outra titulação para confirmar o cloro livre a 2,5%. Em seguida, $Ca(OCl)_2$ a 2,5% e NaOCl a 2,5% foram serialmente diluídos usando um fator de diluição de 1,5 em soro fisiológico (0,9% cloreto de sódio) e aplicados nas células.

Cultura de células e protocolo de tratamento

Culturas permanentes de fibroblastos de camundongo L929 (American Type Culture Collection) e células humanas *osteoblast-like* (Saos-2) foram cultivadas com Meio de Eagle Modificado por Dulbecco (DMEM), suplementado com 10% de soro fetal bovino (SFB) (Gibco por Life Technologies, Paisley, Reino Unido), penicilina a 1% (100 UI/mL) e estreptomicina a 1% (100 mg/mL) (Gibco by Life Technologies), e depois incubados a 37°C, 5% CO_2 e 95% de umidade relativa. As células foram cultivadas em placas de 6, 24 ou 96 poços (Corning, Corning, NY, EUA) ou em lâminas Lab-Tek (Nalge Nunc, Naperville, IL, EUA) contendo DMEM com 10% de SFB e incubadas por 24h. O meio de cultura foi removido e as células foram incubadas com as soluções testadas e controles por 10 min para realizar todos os ensaios e, em seguida, incubadas com DMEM contendo 10% de SFB por 4 h. A solução salina e DMEM foram usados como controles negativos e DMSO a 20%, como controle positivo (Viola et al., 2018; Coaguila-Llerena et al., 2020).

Viabilidade celular por ensaios de metil-tiazol-tetrazólio (MTT) e vermelho neutro (NR)

Os fibroblastos L929 ($1,5 \times 10^4$ células/poço) foram cultivados em placas de 96 poços para ensaios de MTT e NR. No ensaio de MTT, o protocolo de tratamento foi realizado com as soluções a 0,0013% - 0,0225% e os controles, sendo que depois o meio de cultura foi removido, e 100 µL de 1,0 mg/mL da solução de MTT (Sigma-Aldrich, St Louis, MO, EUA) foi adicionado a cada poço. As células foram incubadas a uma atmosfera de 5% de CO_2 e 95% de umidade a 37°C por 3 h. Em seguida, a solução foi removida e foram adicionados 100 µL de álcool isopropílico acidificado (HCl: álcool isopropílico 0,04N). As densidades ópticas das soluções foram medidas usando um espectrofotômetro (Spectramax M, Molecular Devices, San Jose, CA, EUA) no comprimento de onda de 570 nm e analisadas usando um software pertencente ao equipamento (SoftMax Pro, Molecular Devices). A porcentagem de viabilidade celular foi calculada com base no valor de absorbância do controle (soro fisiológico), considerado como 100%.

O ensaio de NR foi realizado utilizando o protocolo de tratamento com as soluções a 0,0013% - 0,0225% e os controles, sendo que depois o meio de cultura foi removido e foi adicionado 100 µL de 0,05 mg/mL de solução de NR (Sigma-Aldrich) a cada poço. As células foram incubadas a atmosfera de 5% de CO_2 e 95% de umidade a 37°C por 3 h. Em seguida, 100 µL de solução de ácido acético a 1% em etanol a 50% foi adicionado a cada poço. As densidades ópticas foram medidas em espectrofotômetro (Spectramax M, Molecular Devices) no comprimento de onda de 570 nm, e analisadas no software pertencente ao equipamento (SoftMax Pro, Molecular Devices). A porcentagem de viabilidade celular foi calculada utilizando-se o valor de absorbância do controle (solução salina), considerado como 100%. Os ensaios de NR e MTT foram realizados em triplicata e repetidos em 3 momentos diferentes.

Análise de morte celular por citometria de fluxo

O protocolo de tratamento foi realizado com as soluções a 0,0013%, 0,0019%, 0,0029%, 0,004%, 0,006% e 0,015%. Na sequência, os fibroblastos L929 (8 x 10^4 células/poço) previamente cultivados em placas de 24 poços foram lavados com solução salina tamponada com fosfato (PBS), e coletada dos poços da placa de cultura usando detergente não enzimático (Gibco por Life Technologies). O peróxido de hidrogênio (1 mM) foi o controle positivo. As células foram incubadas em solução tampão (1x Binding Buffer) contendo anexina V-FITC e iodeto de propídio (PI), de acordo com as instruções do fabricante (eBioscienceTM Annexin V-FITC Apoptosis Detection Kit; Invitrogen by Thermo Fisher Scientific, Viena, Áustria). Pelo menos 1 x 10^5 células por amostra foram adquiridas e analisadas com um classificador de células ativado por fluorescência (FACS; Beckton Dickinson, San Jose, CA, EUA). As populações individualizadas fechadas específicas foram avaliadas com base nos parâmetros de tamanho (FSC) e granularidade (SSC) ou fluorescência (FL). Um total de 20.000 eventos foram analisados para cada amostra. A análise foi realizada de acordo com os seguintes parâmetros: células viáveis (FITC-/PI-), apoptose inicial (FITC+/PI-), apoptose final (FITC+/PI+) e necrose celular (FITC-/PI+). Os resultados foram apresentados como células viáveis, apoptose e necrose (apoptose final + necrose celular). Os experimentos foram realizados em triplicata e repetidos em 2 pontos de tempo diferentes.

Análise do citoesqueleto por fluorescência de actina e α-tubulina

Os fibroblastos L929 (6 x 10^4 células/poço) foram cultivados em lâminas Lab-Tek^{TM} (Nalge Nunc, Rochester, Nova York, EUA), e submetidos ao protocolo de tratamento com soluções a 0,0029%, 0,006% e 0,015%. As células foram lavadas com PBS, fixadas com paraformaldeído a 4% por 20 minutos, lavadas 3 vezes com PBS e permeabilizadas com triton x-100 a 0,5% (Sigma-Aldrich). Os microtúbulos foram marcados com anticorpo α-tubulina (anti-α-tubulina monoclonal de camundongo; Santa Cruz Biotechnology, Santa Cruz, CA, EUA; 1:200) a 4°C durante a noite e, em seguida, com IgG *anti-mouse* conjugado com FITC (Vector Laboratories, Burlingame, CA, EUA; 1:200) a 37°C por 1 h. Os filamentos de actina foram marcados incubando as células com actina (Alexa Fluor® 594 Phalloidin - Molecular Probes, Eugene, OR, EUA) diluída em albumina de soro bovino a 1% (BSA), 1:100, por 30 minutos a 37°C. A marcação de DNA consistiu em incubar as células com DAPI 300 nM (4', 6-diamidino-2-fenilindol, Sigma-Aldrich) por 3 minutos a 37°C. As lâminas foram montadas com Prolong^{TM} (Invitrogen Molecular Probes, Eugene, Oregon, EUA). As células foram analisadas e fotografadas utilizando um microscópio Leica DM 6000M (Leica Microsystems, Wetzlar, Alemanha) acoplado a um Leica AF6000 Deconvolution System (Leica Microsystems).

Análise da ultraestrutura celular por microscopia eletrônica de transmissão (TEM)

Esta avaliação morfológica foi realizada para identificar a organela alvo primária de soluções irrigadoras a 0,0029%, 0,006% e 0,015% em fibroblastos L929 (10 x 10^5 células/poço) cultivados em placas de 6 poços. Os procedimentos foram realizados de acordo com estudo prévio (Faria et al., 2009). Resumidamente, após a aplicação do protocolo de tratamento, as células foram retiradas de cada poço utilizando um *scraper* (Corning), e centrifugadas a 400G por 6 minutos a 20ºC. Em seguida, foram fixadas em aldeído glutárico a 1,4% em tampão cacodilato de sódio 0,1 mol/L com sacarose 0,2 mol/L (pH 7,4) a 37°C por 30 min, e pós-fixadas em tetróxido de ósmio a 1% a 4°C por 1 hora. As células foram lavadas em tampão cacodilato de sódio a 0,1 mol/L e, em seguida, mantidas em solução de acetato de uranila a 0,5% com sacarose por 12 horas a 4°C (contraste em bloco). Em seguida, as células foram desidratadas em concentrações crescentes de acetona e embutidas em resina Araldite® 502 (Polysciences, Warrington, PA, EUA). Cortes ultrafinos foram observados e fotografados usando um microscópio eletrônico de transmissão (EM 109, Carl Zeiss Microscopy GmbH, Oberkochen, Alemanha) a 80 kV.

Atividade da fosfatase alcalina (ALP)

As Saos-2 foram cultivadas em placas de 96 poços a 1 x 10^4 células/poço e incubadas por 24 h. Em seguida, foram incubadas com soluções de $Ca(OCl)_2$ e NaOCl a 0,002%, 0,0002%, 0,00002% e 0,000002% por 1, 3 e 7 dias. O ensaio MTT foi realizado após cada período. As concentrações de 0,00002% e 0,000002% não mostraram efeitos citotóxicos até 7 dias (dados não apresentados); por esta razão, a concentração 0,00002% foi escolhida para avaliar a atividade da ALP. As células foram incubadas com $Ca(OCl)_2$ e NaOCl a 0,00002% por 1, 3 e 7 dias. Após cada período, as células foram incubadas com solução de lauril sulfato de sódio (1 mg/mL) a temperatura ambiente por 30 minutos, e a solução do kit comercial (Labtest, Lagoa Santa, MG, Brasil) foi adicionada seguindo as instruções do fabricante. A densidade óptica das soluções foi medida por um espectrofotômetro (Spectramax M, Molecular Devices) a 590 nm, e a atividade da ALP foi calculada como µmol timolftaleína/min/L/OD. Os dados foram expressos como atividade de ALP normalizada pelo conteúdo de proteínas totais, que também foi determinado com o kit Labtest. O ensaio MTT simultâneo foi realizado em 1, 3 e 7 dias para monitorar a viabilidade celular (Ochoa-Rodríguez et al., 2019).

APPENDIX C – EXTENDED METHODOLOGY (IN PORTUGUESE) OF PUBLICATION 4

“Multispecies biofilm removal by a multisonic irrigation system in mandibular molars”

Seleção de amostra

O cálculo de poder foi realizado em um estudo prévio para determinar o tamanho da amostra necessário (n = 11) para distinguir diferenças entre comunidades pré e pós-tratamento (tomadas como abundância observada de gêneros) usando o pacote HMP no software R (la Rosa et al., 2012; Ordinola-Zapata et al., 2022). Após aprovação do Comitê de Ética (CAAE: 37090820.3.0000.5416) **[ATTACHMENT B]**, foram utilizados 22 molares inferiores humanos permanentes, extraídos por motivos não relacionados a este estudo. Os dentes foram selecionados seguindo os seguintes critérios de inclusão: ápices intactos, sem restaurações extensas, ausência de calcificações, curvatura do canal radicular entre 20 e 40° e configuração de Vertucci tipo II em raízes mesiais confirmadas por micro-CT (Skyscan 1176, Bruker-MicroCT, Kontic, Bélgica). Após realizar a cavidade de acesso e permeabilização, o comprimento de trabalho (a 1 mm do forame apical) foi confirmado usando uma lima K10 (Dentsply, Charlotte, NC, EUA). Em seguida, os dentes foram autoclavados e armazenados em água destilada.

Contaminação do canal radicular

O biofilme multiespécies foi obtido usando uma amostra de placa dental subgengival humana de um único doador saudável (BPE 0-1), que foi obtida de espaços interdentais de molares inferiores usando agulhas de inoculação estéreis (Fisher Scientific, Hampton, NH, EUA), como descrito anteriormente (Ordinola-Zapata et al., 2022). A amostra foi colocada e diluída em meio de transporte anaeróbico (Anaerobe System, Morgan Hills, CA, EUA). Os canais radiculares foram revestidos primeiro com 0,1mL de sangue de carneiro (HemoStat Laboratories, Dixon, CA, EUA) para promover a formação de uma camada orgânica e, em seguida, foram introduzidos 100µL de placa dental diluída. Os dentes foram montados em um suporte personalizado. Este suporte foi colocado no reator *Center for Disease Control* (CDC) (BioSurface Technologies, Bozeman, MT, EUA), que produz condições para um modelo de microcosmo oral (Fig. 2). Todos os componentes do reator foram autoclavados. O reator possui um recipiente com tampa que permite um fluxo definido de 350mL de meio Columbia, (Difco, BD, Sparks, MD, EUA), e possui uma barra de agitação que gera forças de agitação (Rudney et al., 2012). A temperatura interna do recipiente CDC foi ajustada a 37°C e taxa de agitação a 90 rpm. Inicialmente, o reator foi incubado em condições de agitação, sem fluxo de meio, por 24h; em seguida, o meio Columbia foi bombeado para o reator a uma taxa de fluxo de 2,5L/24h por 2 semanas. A presença de uma camada de biofilme amorfo cobrindo diversos substratos, incluindo a superfície da parede do canal radicular, foi previamente validada (Rudney et al., 2012; Li et al., 2014; Ordinola-Zapata et al., 2022). Os dentes foram removidos para tratamento.

Preparo do canal radicular, protocolos finais de irrigação e amostragem microbiana

A superfície externa das coroas dos dentes acessados foi descontaminada com hipoclorito de sódio (NaOCl) a 6% (Clorox, Oakland, CA, EUA) por 3 min e depois com tiossulfato de sódio a 10% por 3 min. O protocolo de descontaminação foi previamente verificado em um estudo piloto confirmando a remoção do DNA bacteriano (dados não mostrados). Na sequência, os canais mesiais foram instrumentados com limas Hedstrom tamanho 15 (Dentsply Maillefer, Ballaigues, Suíça) usando um movimento de vaivém por 30s para gerar raspas de dentina. Em seguida, pontas de papel estéril foram colocados para absorver o conteúdo do canal radicular com as raspas (amostra pré-operatória). Os dentes foram distribuídos aleatoriamente em dois grupos: GentleWave (n=11) e PUI (n=11). Para o grupo GentleWave, foi realizada uma instrumentação mínima com uma lima 20.06 (V-Taper, SSWhite, Lakewood, NJ, EUA) seguindo as recomendações do fabricante. Para o grupo PUI, foi realizada instrumentação convencional até lima 35.04 (Vortex Blue, Dentsply). Todos os canais foram irrigados com 10mL de NaOCl a 3% (Clorox, diluído e titulado a partir de uma solução de NaOCl a 6%) durante o processo de instrumentação usando uma seringa de 3mL e uma agulha de saída lateral 30G a 2-3 mm do comprimento de trabalho. Todos os ápices foram selados com cianoacrilato e cera vermelha estéril para obter um sistema fechado para irrigação. Os protocolos de agitação foram:

Grupo PUI: para irrigação final, NaOCl 3% foi ativado em cada canal (MV e ML), a 2 mm do comprimento de trabalho, utilizando uma ponta ultrassônica 20.02 acoplada a um aparelho piezoelétrico (EndoUltra, Vista Dental, Racine, WI, EUA) na frequência de 40.000 Hz. Dois mL da solução foram ativados por 20s, sendo que o procedimento foi repetido três vezes para um tempo total de irrigação final de 1 minuto. O volume total de NaOCl por canal foi de 6mL. O mesmo procedimento foi repetido para EDTA 17%.

Grupo GentleWave: A cavidade de acesso de todos os dentes foi selada com uma barreira (Soundseal, Sonendo). O assoalho da câmara pulpar foi calibrado para ter a ponta do instrumento de procedimento 1-2mm acima do assoalho pulpar. O protocolo de irrigação consistiu em um ciclo de 1 min com água destilada, um ciclo de 4 min com NaOCl a 3%, um ciclo de 1 min com EDTA a 8,5% e finalmente um ciclo de 1 min com água destilada.

Após a irrigação final, todos os canais foram irrigados com 2mL de tiossulfato de sódio a 10% por 3min para inativar o efeito residual do NaOCl. As amostras microbiológicas pós-operatórias de ambos os grupos foram obtidas usando limas Hedstroem tamanho 15 em movimento de vaivém por 30s (Dentsply, Maillefer). Para a obtenção das amostras foram utilizadas pontas de papel estéril (amostra pós-operatória). As amostras pré e pós-operatórias (pontas de papel) foram colocadas em meio de transporte anaeróbico (Anaerobe System, Morgan Hills, CA, EUA) e agitadas em vórtex por 10s.

Análise quantitativa de PCR em tempo real (qPCR), extração de DNA e análise de sequenciamento

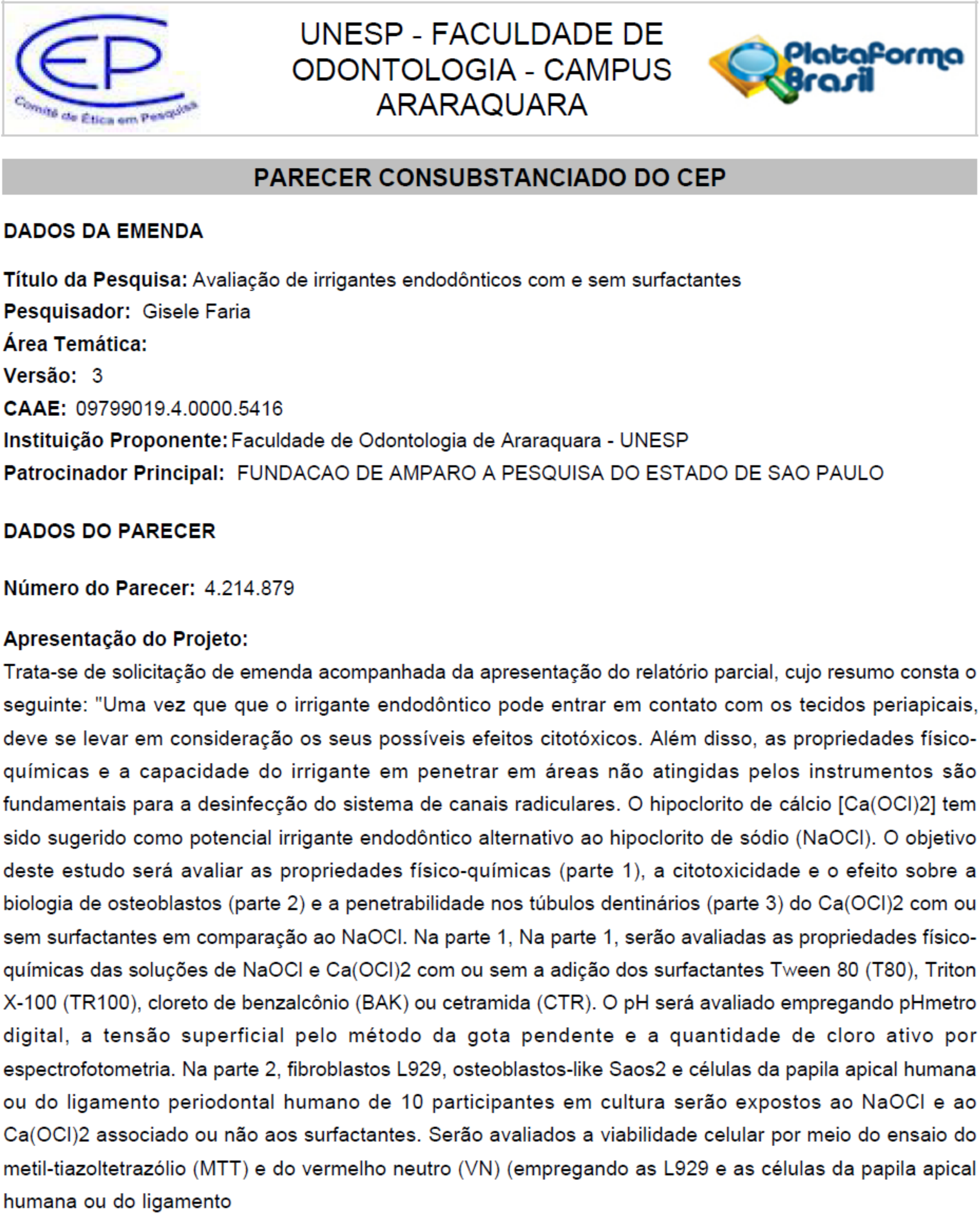
O kit DNeasy® PowerSoil® Pro (Qiagen, Hilden, Alemanha) foi usado para extrair o DNA. O Centro Genômico da instituição processou as amostras para quantificação de qPCR e os resultados foram expressos em moléculas/µL. Para sequenciamento de DNA, a região hipervariável V3-V4 do gene de RNA ribossômico 16S (rRNA) foi amplificada e sequenciada usando sequenciamento de extremidade pareada a um comprimento de leitura de 301 nucleotídeos (nt) na plataforma *Illumina MiSeq* pelo método de índice duplo. Os dados brutos foram obtidos como arquivos ".fastq" e carregados no *Sequence Read Archive* sob o número de acesso do *BioProject* SRP328673.

Processamento e análise de Amplificação

Os dados de sequência foram processados e analisados usando o software *mothur* ver. 1.41.1 (Schloss 2020). As sequências foram primeiro aparadas para os primeiros 250 nt e unidas em pares usando o software fastq-join. O corte de qualidade foi realizado a um limiar de 35 sobre uma janela deslizante de 50 nt. Além disso, sequências com homopolímeros >8 nt, bases ambíguas ou >2 incompatibilidades de sequências dos *primers* foram removidas. As sequências de alta qualidade foram alinhadas com o banco de dados SILVA ver. 138.1 para o processamento. As quimeras foram identificadas e removidas usando UCHIME ver. 4.2.40. Os erros de sequenciamento foram removidos usando uma etapa de pré-agrupamento de 2%. As unidades taxonômicas operacionais (OTUs) foram agrupadas com uma similaridade de 99% usando o algoritmo do vizinho mais distante e foram classificadas em relação à versão 18 do *Ribossomal Database Project* (Cole et al., 2009). Diferentes bases de dados foram usadas para alinhamento e classificação devido às considerações de processamento descritas anteriormente (Schloss & Westcott 2011).

ATTACHMENT A - APPROVAL OF RESEARCH ETHICS COMMITTEE REGARDING OF THE ENTITLED PUBLICATION 1

“Physicochemical properties and penetration into dentinal tubules of calcium hypochlorite with surfactants”



CEP Comitê de Ética em Pesquisa

UNESP - FACULDADE DE
ODONTOLOGIA - CAMPUS
ARARAQUARA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação de irrigantes endodônticos com e sem surfactantes
Pesquisador: Gisele Faria
Área Temática:
Versão: 3
CAAE: 09799019.4.0000.5416
Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 4.214.879

Apresentação do Projeto:

Trata-se de solicitação de emenda acompanhada da apresentação do relatório parcial, cujo resumo consta o seguinte: "Uma vez que que o irrigante endodôntico pode entrar em contato com os tecidos periapicais, deve se levar em consideração os seus possíveis efeitos citotóxicos. Além disso, as propriedades físico-químicas e a capacidade do irrigante em penetrar em áreas não atingidas pelos instrumentos são fundamentais para a desinfecção do sistema de canais radiculares. O hipoclorito de cálcio [$Ca(OCl)2$] tem sido sugerido como potencial irrigante endodôntico alternativo ao hipoclorito de sódio (NaOCl). O objetivo deste estudo será avaliar as propriedades físico-químicas (parte 1), a citotoxicidade e o efeito sobre a biologia de osteoblastos (parte 2) e a penetrabilidade nos túbulos dentinários (parte 3) do $Ca(OCl)2$ com ou sem surfactantes em comparação ao NaOCl. Na parte 1, Na parte 1, serão avaliadas as propriedades físico-químicas das soluções de NaOCl e $Ca(OCl)2$ com ou sem a adição dos surfactantes Tween 80 (T80), Triton X-100 (TR100), cloreto de benzalcônio (BAK) ou cetramida (CTR). O pH será avaliado empregando pHmetro digital, a tensão superficial pelo método da gota pendente e a quantidade de cloro ativo por espectrofotometria. Na parte 2, fibroblastos L929, osteoblastos-like Saos2 e células da papila apical humana ou do ligamento periodontal humano de 10 participantes em cultura serão expostos ao NaOCl e ao $Ca(OCl)2$ associado ou não aos surfactantes. Serão avaliados a viabilidade celular por meio do ensaio do metil-tiazoltetrazólio (MTT) e do vermelho neutro (VN) (empregando as L929 e as células da papila apical humana ou do ligamento periodontal humano), o citoesqueleto por meio de marcação para actina, o tipo de morte celular por citometria de fluxo, a ultraestrutura por microscopia eletrônica de transmissão (empegando L929) e a atividade da fosfatase alcalina pelo cálculo da liberação timolftaleína (empregando as Saos-2). Na parte 3, para análise da penetrabilidade dos irrigantes na dentina, raízes de dentes unirradiculados humanos (n=75), doados pelo banco de dentes da Faculdade de Odontologia de Araraquara - UNESP serão instrumentadas e coradas com cristal violeta e irrigadas com NaOCl ou Ca(OCl)2 associado ou não aos surfactantes e analisadas em estereomicróscopio. Alternativamente, se as condições da pandemia de COVID 19 permitirem, não utilizaremos a metodologia do cristal violeta, mas sim a microscopia confocal de varredura a laser (CLSM). As soluções serão marcadas com fluorocromo para a análise da sua penetrabilidade nos túbulos dentinários em CLSM. A penetrabilidade dos irrigantes na dentina será avaliada nos terços radiculares cervical, médio e apical. A análise estatística dos resultados será efetuada empregando nível de significância de 5%. Os testes estatísticos serão escolhidos de acordo com a distribuição e homocedasticidade dos dados. Em última instância, é nossa expectativa obter informações para entender mais profundamente o mecanismo ação do Ca(OCl)2 nas células eucarióticas, suas propriedades físico-químicas e penetrabilidade nos túbulos dentinários, para fornecer subsídios para a sua indicação ou não na clínica endodôntica.

Objetivo da Pesquisa:

O objetivo deste estudo será avaliar as propriedades físico-químicas (parte 1), o efeito sobre fibroblastos L929, células da papila apical humana ou ligamento periodontal humano, e sobre osteoblastos-like Saos-2 (parte 2), e a penetrabilidade nos túbulos dentinários (parte 3) do Ca(OCl)2, associado ou não a surfactantes, em comparação ao NaOCl.

Avaliação dos Riscos e Benefícios:

Riscos: Para avaliação da penetrabilidade dos irrigantes nos túbulos dentinários, os riscos são mínimos, uma vez que serão utilizados dentes extraídos obtidos do banco de dentes da FOAr – UNESP. No entanto, o pesquisador irá utilizar equipamento de proteção individual (EPI) adequado e seguirá as normas de biossegurança quando for entrar em contato com os dentes e com as soluções para evitar contaminação e/ou acidentes com as soluções. Para realização do exame radiográfico com a finalidade de selecionar os dentes do banco de dentes, eles serão colocados em uma caixa revestida de chumbo para proteção da radiação. Esta caixa será disponibilizada em um dos laboratórios do Departamento onde será realizada a pesquisa. Para os ensaios de citotoxicidade, a coleta da papila apical e/ou do ligamento periodontal não oferece riscos para o paciente, uma vez que a mesma será realizada após extração dentária. Em relação à extração dentária propriamente dita, ela será realizada por residentes da disciplina de Cirurgia e Traumatologia Buco-Maxilo-Facial da Faculdade de Odontologia de Araraquara – UNESP, que são treinados para realizar o procedimento. Caso os participantes apresentem complicações pós-operatórias como edema, dor, infecção, alveolite, entre outras, eles serão atendidos pelos residentes, que tomarão as providências para solucionar os problemas. Para realização de todos os procedimentos, serão asseguradas as normas da Biossegurança da Faculdade de Odontologia de Araraquara – UNESP.

Benefícios: Os benefícios não serão diretos para os participantes, mas sim indiretos, uma vez que os resultados do estudo permitirão obter um maior conhecimento do hipoclorito de cálcio com ou sem surfactantes, fornecendo subsídios para a sua indicação ou não na clínica endodôntica.

Comentários e Considerações sobre a Pesquisa:
Estudo nacional, prospectivo, randomizado, de caráter acadêmico a ser realizado para obtenção de título de doutor. Utilizará recursos do projeto FAPESP nº 2018/24662-6. Número de participantes incluídos no Brasil: 75 dentes provenientes do Banco de Dentes e 10 participantes que doarão células da papila apical humana ou do ligamento periodontal humano. Não ocorrerá armazenamento de amostras em banco de material biológico no Brasil. Estudo já iniciou e tem previsão de encerramento em 30/09/2022.
Critério de Inclusão: Para avaliação da penetrabilidade dos irrigantes nos túbulos dentinários serão usados dentes doados pelo do banco de dentes da FOAr – UNESP. Estes dentes deverão ser unirradiculados e sem calcificação, reabsorção interna/externa, ápices imaturos, lesão de cárie, trincas, fraturas na superfície radicular e devem apresentar curvatura do canal radicular até 10°. Para avaliação da citotoxicidade dos irrigantes serão obtidos os tecidos da papila apical e/ou do ligamento periodontal de terceiros molares extraídos por razões ortodônticas, sem cárie ou doença periodontal e com rizogênese incompleta, de participantes jovens e saudáveis com idade entre 16-25 anos, assegurando as normas da Biossegurança da Faculdade de Odontologia de Araraquara – UNESP.
Justificativa da solicitação de emenda segundo o pesquisador responsável: alteração de metodologia, do número de sujeitos da pesquisa e prorrogação do prazo de vigência pelos motivos abaixo relacionados:
"(1) Pretende-se alterar a metodologia do item “penetrabilidade dos irrigantes nos túbulos dentinários”, que compreendia, no projeto inicialmente aprovado pelo CEP, a análise por estereomicroscópio da extensão da dentina corada com violeta cristal, que seria

branqueada/descorada pelos os irrigantes. Pretendemos empregar a metodologia de análise da penetração dos irrigantes na dentina por microscópio confocal de varredura a laser (CLSM), que tem sido mais comumente relatada na literatura (Llena et al., 2015; Akcay et al., 2017; Gu et al., 2017).

Esta alteração de metodologia não exigirá dentes humanos extraídos adicionais aos 75 já aprovados pelo CEP.

Salienta-se que para a realização do ensaio de "penetrabilidade dos irrigantes na dentina", empregando o CLSM, estaremos sujeitos às normas da UNESP e da FOAr que, em função da pandemia de COVID-19, regulamentam as atividades da pós-graduação e o uso de laboratórios multiusuário. Considerando que não sabemos quando o CLSM poderá ser utilizado e qual o número de usuários já agendados, poderemos voltar ao nosso planejamento inicial, aprovado pelo CEP, que consiste na utilização de pigmentação dos dentes com cristal violeta e análise por estereomicroscópio da região descorada pelos irrigantes.

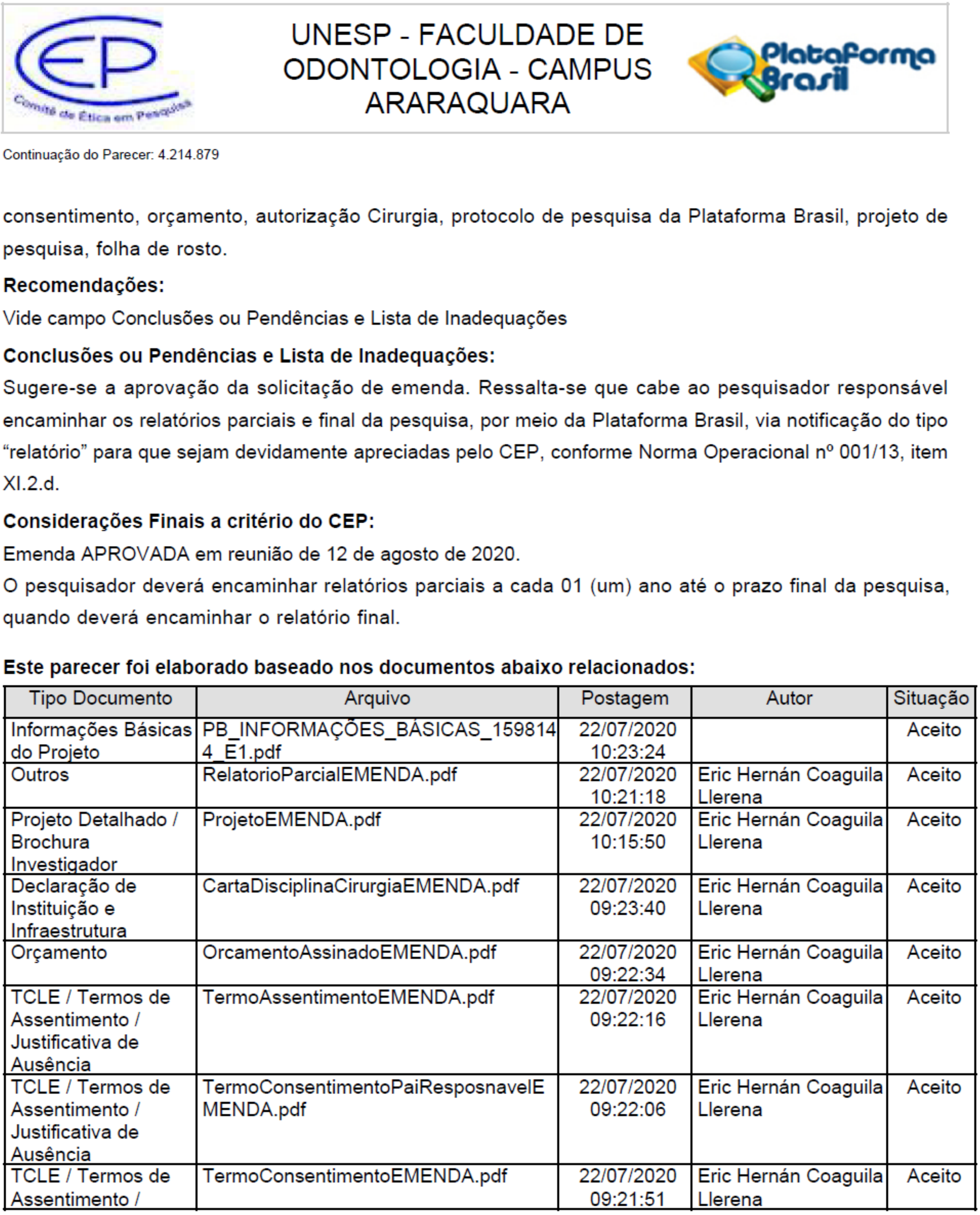
(2) Pretendemos adicionar ao projeto de pesquisa outros ensaios que são avaliação da citotoxicidade das soluções por meio dos testes de metil-tiazol-tetrazolio (MTT) e vermelho neutro, empregando células da papila apical de dentes humanos. A vantagem do uso destas células, é que elas são as principais que participam do processo de revitalização pulpar de dentes com necrose pulpar e rizogênese incompleta. No entanto, se não for possível coletar a papila apical devido a pequena quantidade de exodontia de terceiros molares com rizogênese incompleta (que apresentam papila apical) que aparecerem na clínica de cirurgia da FOAr ou devido à "perda" da papila apical durante a cirurgia, iremos coletar células do ligamento periodontal para avaliação da citotoxicidade das soluções irrigadoras, o que não levará a nenhum prejuízo para os pacientes. As células do ligamento também participam dos processos de reparação periapical e de revitalização pulpar. Os ensaios de citotoxicidade serão realizados em triplicata, utilizando células de 3 doadores. No entanto, fizemos a previsão de coletar as células de dentes de 10 pacientes, devido a perdas que podem ocorrer devido à contaminação da cultura celular ou à não proliferação das células em cultura, para que ao final tenhamos as células de pelo menos 3 doadores.

(3) A prorrogação da vigência do projeto de pesquisa, de 30/09/2021 até 30/09/2022, será necessária para finalizar os ensaios, considerando o período de isolamento social devido à pandemia de COVID-19 e à alteração da metodologia.

Obs: As alterações realizadas no projeto de pesquisa estavam destacadas em vermelho."

Considerações sobre os Termos de apresentação obrigatória:

Termos obrigatórios apresentados: relatório parcial, termo de assentimento, termo de



consentimento, orçamento, autorização Cirurgia, protocolo de pesquisa da Plataforma Brasil, projeto de pesquisa, folha de rosto.

Recomendações:

Vide campo Conclusões ou Pendências e Lista de Inadequações

Conclusões ou Pendências e Lista de Inadequações:

Sugere-se a aprovação da solicitação de emenda. Ressalta-se que cabe ao pesquisador responsável encaminhar os relatórios parciais e final da pesquisa, por meio da Plataforma Brasil, via notificação do tipo "relatório" para que sejam devidamente apreciadas pelo CEP, conforme Norma Operacional nº 001/13, item XI.2.d.

Considerações Finais a critério do CEP:

Emenda APROVADA em reunião de 12 de agosto de 2020.

O pesquisador deverá encaminhar relatórios parciais a cada 01 (um) ano até o prazo final da pesquisa, quando deverá encaminhar o relatório final.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1598144_E1.pdf	22/07/2020 10:23:24		Aceito
Outros	RelatorioParcialEMENDA.pdf	22/07/2020 10:21:18	Eric Hernán Coaguila Llerena	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoEMENDA.pdf	22/07/2020 10:15:50	Eric Hernán Coaguila Llerena	Aceito
Declaração de Instituição e Infraestrutura	CartaDisciplinaCirurgiaEMENDA.pdf	22/07/2020 09:23:40	Eric Hernán Coaguila Llerena	Aceito
Orçamento	OrcamentoAssinadoEMENDA.pdf	22/07/2020 09:22:34	Eric Hernán Coaguila Llerena	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TermoAssentimentoEMENDA.pdf	22/07/2020 09:22:16	Eric Hernán Coaguila Llerena	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TermoConsentimentoPaiResposnavelEMENDA.pdf	22/07/2020 09:22:06	Eric Hernán Coaguila Llerena	Aceito
TCLE / Termos de Assentimento /	TermoConsentimentoEMENDA.pdf	22/07/2020 09:21:51	Eric Hernán Coaguila Llerena	Aceito

UNESP - FACULDADE DE ODONTOLOGIA - CAMPUS ARARAQUARA

Continuação do Parecer: 4.214.879

Justificativa de Ausência	TermoConsentimentoEMENDA.pdf	22/07/2020 09:21:51	Eric Hernán Coaguila Llerena	Aceito
Folha de Rosto	FolhaderostoEMENDA.pdf	20/07/2020 23:08:59	Eric Hernán Coaguila Llerena	Aceito
Outros	RespostaCEP.pdf	21/05/2019 16:48:33	Eric Hernán Coaguila Llerena	Aceito
Outros	Declaracao_de_nao_ressarcimento.pdf	11/03/2019 18:42:21	Eric Hernán Coaguila Llerena	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Dispensa_TCLE.pdf	11/03/2019 18:40:01	Eric Hernán Coaguila Llerena	Aceito
Declaração de Pesquisadores	Declaracao_do_pesquisador.pdf	11/03/2019 18:39:08	Eric Hernán Coaguila Llerena	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	BancoDentes.pdf	11/03/2019 18:38:55	Eric Hernán Coaguila Llerena	Aceito
Declaração de Instituição e Infraestrutura	Infraestrutura2.pdf	11/03/2019 18:33:54	Eric Hernán Coaguila Llerena	Aceito
Declaração de Instituição e Infraestrutura	Infraestrutura.pdf	11/03/2019 18:33:45	Eric Hernán Coaguila Llerena	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

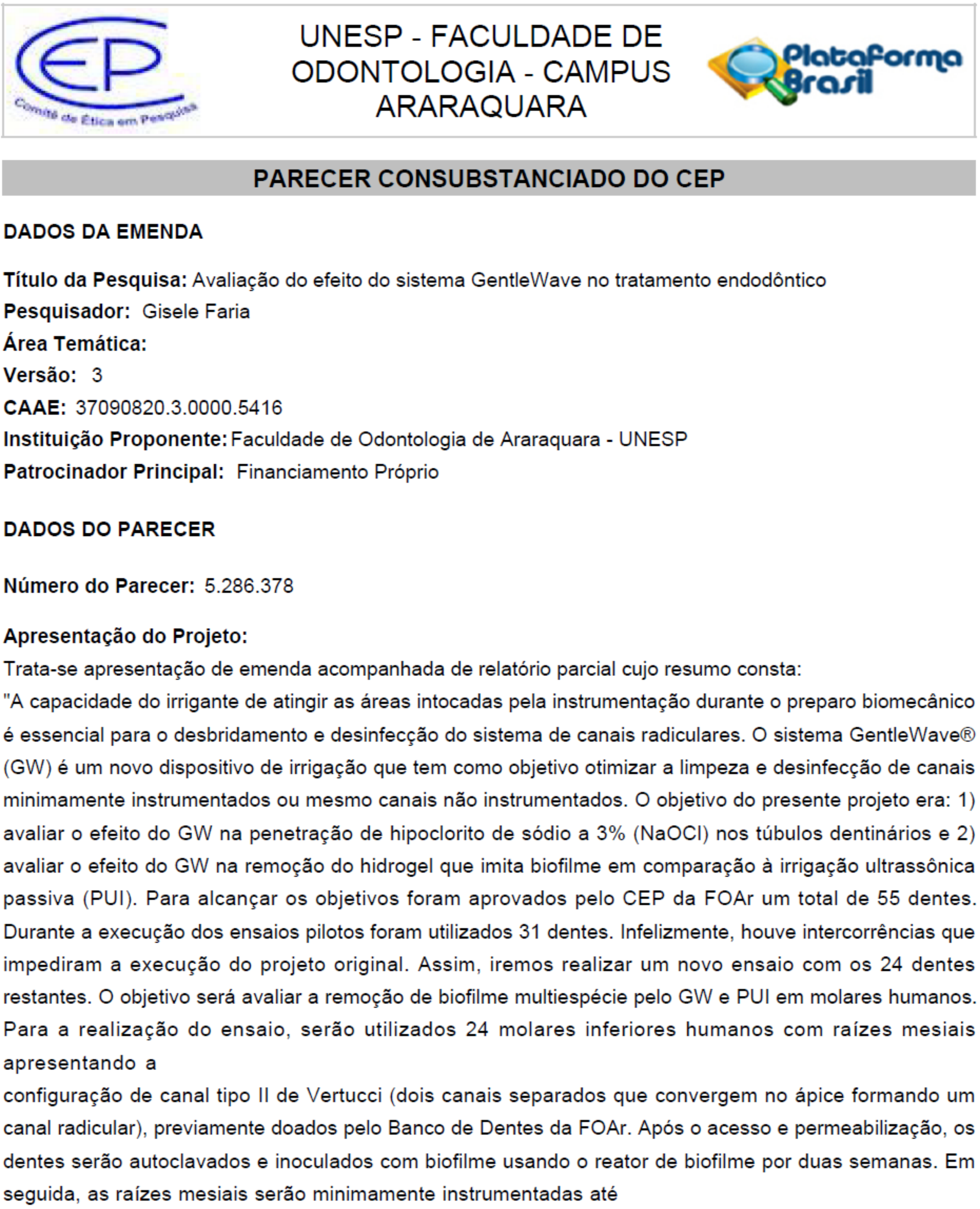
Não

ARARAQUARA, 15 de Agosto de 2020

Assinado por:
Andréa Gonçalves
(Coordenador(a))

ATTACHMENT B - APPROVAL OF RESEARCH ETHICS COMMITTEE REGARDING OF THE ENTITLED PUBLICATION 4

“Multispecies biofilm removal by a multisonic irrigation system in mandibular molars”



UNESP - FACULDADE DE ODONTOLOGIA - CAMPUS ARARAQUARA

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação do efeito do sistema GentleWave no tratamento endodôntico
Pesquisador: Gisele Faria
Área Temática:
Versão: 3
CAAE: 37090820.3.0000.5416
Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.286.378

Apresentação do Projeto:
Trata-se apresentação de emenda acompanhada de relatório parcial cujo resumo consta:
"A capacidade do irrigante de atingir as áreas intocadas pela instrumentação durante o preparo biomecânico é essencial para o desbridamento e desinfecção do sistema de canais radiculares. O sistema GentleWave® (GW) é um novo dispositivo de irrigação que tem como objetivo otimizar a limpeza e desinfecção de canais minimamente instrumentados ou mesmo canais não instrumentados. O objetivo do presente projeto era: 1) avaliar o efeito do GW na penetração de hipoclorito de sódio a 3% (NaOCl) nos túbulos dentinários e 2) avaliar o efeito do GW na remoção do hidrogel que imita biofilme em comparação à irrigação ultrassônica passiva (PUI). Para alcançar os objetivos foram aprovados pelo CEP da FOAr um total de 55 dentes. Durante a execução dos ensaios pilotos foram utilizados 31 dentes. Infelizmente, houve intercorrências que impediram a execução do projeto original. Assim, iremos realizar um novo ensaio com os 24 dentes restantes. O objetivo será avaliar a remoção de biofilme multiespécie pelo GW e PUI em molares humanos. Para a realização do ensaio, serão utilizados 24 molares inferiores humanos com raízes mesiais apresentando a
configuração de canal tipo II de Vertucci (dois canais separados que convergem no ápice formando um canal radicular), previamente doados pelo Banco de Dentes da FOAr. Após o acesso e permeabilização, os dentes serão autoclavados e inoculados com biofilme usando o reator de biofilme por duas semanas. Em seguida, as raízes mesiais serão minimamente instrumentadas até

a lima 20.06 para o grupo GW, e convencionalmente instrumentadas até a lima 35.04 para o grupo PUI, sob irrigação com NaOCl 3%. A irrigação final será realizada com GW e PUI (n = 12 por grupo). Amostras serão analisadas utilizando a técnica da reação em cadeia da polimerase (qPCR) em tempo real e sequenciamento do gene do RNA ribossômico 16S (Next Generation Sequencing - NGS). Os testes estatísticos serão escolhidos de acordo com a distribuição dos dados e homocedasticidade."

Objetivo da Pesquisa:

O objetivo do presente projeto será avaliar a remoção de biofilme multiespécie pelo sistema GentleWave® (GW) e irrigação ultrassônica passiva (PUI) em molares humanos.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos desta pesquisa são mínimos, uma vez que serão utilizados dentes extraídos doados pelo banco de dentes da FOAr – UNESP. No entanto, os pesquisadores irão utilizar equipamentos de proteção individual (EPI) adequados e seguirão as normas de biossegurança da instituição, quando forem entrar em contato com os dentes, com o biofilme e com as soluções para evitar contaminação e/ou acidentes. Para a avaliação do ângulo e

raio de curvatura radicular no projeto original, os dentes foram colocados em uma caixa revestida de chumbo durante as tomadas radiográficas. Esta caixa foi disponibilizada no Laboratório de Pesquisa em Endodontia – centro de confecção de corpos de prova do Departamento de Odontologia Restauradora.

Benefícios:

O benefício principal é obter informações que permitam maior conhecimento do sistema GentleWave, e fornecer subsídios para a sua indicação ou não na clínica endodôntica.

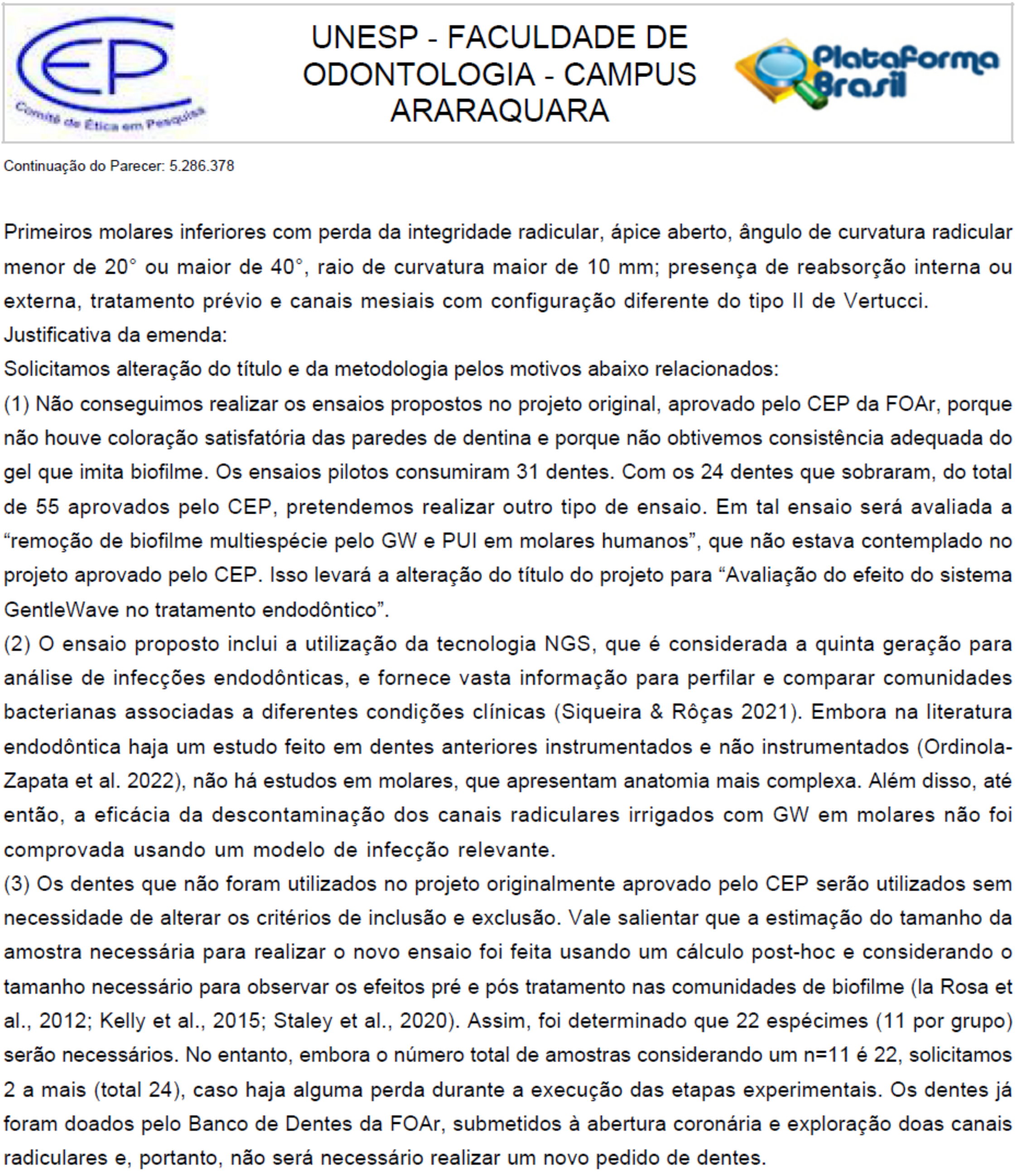
Comentários e Considerações sobre a Pesquisa:

Trata-se de estudo experimental laboratorial, pois haverá intervenção, distribuição aleatória dos sujeitos (dentes) aos grupos da pesquisa e grupo controle.

Critério de Inclusão:

Primeiros molares inferiores humanos com integridade radicular, ápice fechado, ângulo de curvatura radicular entre 20° e 40° e raio de curvatura menor de 10 mm, ausência de reabsorção interna ou externa, nenhum tratamento prévio e com configuração tipo II de Vertucci nos canais mesiais.

Critério de Exclusão:



Primeiros molares inferiores com perda da integridade radicular, ápice aberto, ângulo de curvatura radicular menor de 20° ou maior de 40°, raio de curvatura maior de 10 mm; presença de reabsorção interna ou externa, tratamento prévio e canais mesiais com configuração diferente do tipo II de Vertucci.

Justificativa da emenda:

Solicitamos alteração do título e da metodologia pelos motivos abaixo relacionados:

(1) Não conseguimos realizar os ensaios propostos no projeto original, aprovado pelo CEP da FOAr, porque não houve coloração satisfatória das paredes de dentina e porque não obtivemos consistência adequada do gel que imita biofilme. Os ensaios pilotos consumiram 31 dentes. Com os 24 dentes que sobraram, do total de 55 aprovados pelo CEP, pretendemos realizar outro tipo de ensaio. Em tal ensaio será avaliada a “remoção de biofilme multiespécie pelo GW e PUI em molares humanos”, que não estava contemplado no projeto aprovado pelo CEP. Isso levará a alteração do título do projeto para “Avaliação do efeito do sistema GentleWave no tratamento endodôntico”.

(2) O ensaio proposto inclui a utilização da tecnologia NGS, que é considerada a quinta geração para análise de infecções endodônticas, e fornece vasta informação para perfilar e comparar comunidades bacterianas associadas a diferentes condições clínicas (Siqueira & Rôças 2021). Embora na literatura endodôntica haja um estudo feito em dentes anteriores instrumentados e não instrumentados (Ordinola-Zapata et al. 2022), não há estudos em molares, que apresentam anatomia mais complexa. Além disso, até então, a eficácia da descontaminação dos canais radiculares irrigados com GW em molares não foi comprovada usando um modelo de infecção relevante.

(3) Os dentes que não foram utilizados no projeto originalmente aprovado pelo CEP serão utilizados sem necessidade de alterar os critérios de inclusão e exclusão. Vale salientar que a estimação do tamanho da amostra necessária para realizar o novo ensaio foi feita usando um cálculo post-hoc e considerando o tamanho necessário para observar os efeitos pré e pós tratamento nas comunidades de biofilme (la Rosa et al., 2012; Kelly et al., 2015; Staley et al., 2020). Assim, foi determinado que 22 espécimes (11 por grupo) serão necessários. No entanto, embora o número total de amostras considerando um n=11 é 22, solicitamos 2 a mais (total 24), caso haja alguma perda durante a execução das etapas experimentais. Os dentes já foram doados pelo Banco de Dentes da FOAr, submetidos à abertura coronária e exploração doas canais radiculares e, portanto, não será necessário realizar um novo pedido de dentes.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados os seguintes documentos, atualizados de acordo com a solicitação de emenda: projeto de pesquisa, protocolo PB Informações, termo de responsabilidade do pesquisador responsável, solicitação de dispensa do CLE e relatório parcial.

Recomendações:

Vide campo conclusões ou pendências e lista de inadequações.

Conclusões ou Pendências e Lista de Inadequações:

Sugere-se a aprovação da emenda acompanhada de relatório parcial.

Considerações Finais a critério do CEP:

Emenda APROVADA em reunião de 11 de março de 2022.

O pesquisador deverá encaminhar relatório parcial no meio do período da pesquisa até o prazo final da pesquisa, quando deverá encaminhar o relatório final.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1905688_E1.pdf	28/02/2022 16:57:46		Aceito
Outros	Dispensa_TCLE_EMENDA.pdf	28/02/2022 16:45:57	Eric Hernán Coaguila Llerena	Aceito
Declaração de Pesquisadores	Declaracao_pesquisador_EMENDA.pdf	28/02/2022 16:44:52	Eric Hernán Coaguila Llerena	Aceito
Outros	Acceptance_Letter_EMENDA.pdf	28/02/2022 16:44:17	Eric Hernán Coaguila Llerena	Aceito
Outros	RelatorioParcialEMENDA_GW.pdf	28/02/2022 16:43:04	Eric Hernán Coaguila Llerena	Aceito
Outros	Projeto_original.pdf	28/02/2022 16:42:18	Eric Hernán Coaguila Llerena	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_GW_EMENDA.pdf	28/02/2022 16:41:03	Eric Hernán Coaguila Llerena	Aceito
Outros	Carta_Resposta_CEP_R1.pdf	12/09/2020 22:54:06	Eric Hernán Coaguila Llerena	Aceito
Outros	AcceptanceLetter.pdf	24/08/2020 14:03:21	Eric Hernán Coaguila Llerena	Aceito
Declaração de	Declaracao_banco_de_dentesASSINA	24/08/2020	Eric Hernán	Aceito

Manuseio Material Biológico / Biorepositório / Biobanco	DA.pdf	14:02:34	Coaguila Llerena	Aceito
Orçamento	Orcamento.pdf	21/08/2020 13:13:00	Eric Hernán Coaguila Llerena	Aceito
Declaração de Instituição e Infraestrutura	InfraestruturaLabEndo.pdf	21/08/2020 13:12:15	Eric Hernán Coaguila Llerena	Aceito
Outros	DispensaTCLE.pdf	21/08/2020 13:12:01	Eric Hernán Coaguila Llerena	Aceito
Declaração de Pesquisadores	DeclaracaoPesquisador.pdf	21/08/2020 13:09:07	Eric Hernán Coaguila Llerena	Aceito
Folha de Rosto	FolhaDeRostoAssinada.pdf	21/08/2020 13:08:44	Eric Hernán Coaguila Llerena	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARARAQUARA, 11 de Março de 2022

Assinado por:
Andréa Gonçalves
(Coordenador(a))

Araraquara, 5 de setembro de 2022.

Eric Hernán Coaguila Llerena