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Research Article

Antimicrobial Effect of Calcium Hydroxide Combined with Electrolyzed Superoxidized Solution at Neutral pH on *Enterococcus faecalis* Growth

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Objective. To evaluate the effect of the combination of calcium hydroxide ($Ca(OH)_2$) and a novel electrolyzed superoxidized solution at neutral pH, known as OxOral® on *Enterococcus faecalis* growth in root canals. *Methods*. Sixty human teeth were used, from which root canals were infected and randomly divided into the following treatment groups: saline solution, saline solution plus $Ca(OH)_2$, OxOral®, and OxOral® plus $Ca(OH)_2$. *Results*. A permanent reduction in bacterial growth was observed at days 1, 6, 12, and 18 after OxOral® plus $Ca(OH)_2$ treatment from 4.4 ± 0.074 $\log_{10}$ CFU/mL to 0.0 ± 0.001 $\log_{10}$ CFU/mL. In addition, alkaline conditions maintenance was observed from application time ($pH = 12.2 \pm 0.033$) to 18 d posttreatment ($pH = 12.6 \pm 0.083$). *Conclusion*. The combination of OxOral® and $Ca(OH)_2$ provides an alkaline pH and inhibits *E. faecalis* growth into the root canals. Our study opens the possibility for further research on the use of OxOral® in endodontic therapy.

1. Introduction

Untreated dental caries, crown, or root fractures of the tooth, conservative dental treatments that have not been properly applied, and exposure of pulp tissue to chemical agents or dental materials may lead to inflammation of the dental pulp tissue, known as pulpitis. Its progression leads to an irreversible condition, where the inflammation spreads to the tooth apices, periodontium, dentinal tubules, and blood vessels [1].

The endodontic treatment is commonly recommended, when there is irreversible pulpitis or pulp necrosis. Therapy consists of the total removal of the dental pulp and the three-dimensional sealing of the root canal to restore the function of the teeth within the masticatory apparatus [2, 3]. During this treatment, the root canal is provisionally sealed with calcium hydroxide ($Ca(OH)_2$), which may be dissolved in saline solution or anesthesia, to keep clean and aseptic [3–5]. $Ca(OH)_2$ is an odorless white powder obtained by calcination of calcium carbonate and its transformation into calcium oxide, which possesses a molecular weight of 74.08 g/mol, low solubility in water with a pH = 12.5 – 12.8, and is insoluble in alcohol. Many studies have demonstrated the properties

of $Ca(OH)_2$-based sealants, such as physical properties, biocompatibility, leaks, adhesion, antibacterial, and periapical healing effects [6, 7]. The two most important reasons for using $Ca(OH)_2$ in a dressing or intracanal medication are stimulation of the periapical tissues to maintain health and promotion of healing and its antimicrobial effects. Although the exact mechanisms are unknown, the following ones have been proposed: (a) $Ca(OH)_2$ is antibacterial depending on the availability of free hydroxyl ions that encourages repair and active calcification [8–10]. Furthermore, it has been suggested that it acts indirectly by obliterating the root canal space, thus limiting the use of nutrients by the microorganisms lodged in the dentin [11], (b) ?added value="t"?>he alkaline pH neutralizes lactic acid from osteoclasts and prevents dissolution of mineralized components of teeth and also activates alkaline phosphatase and calcium-dependent adenosine triphosphatase reaction leading to hard tissue formation [12, 13], (c) it denatures proteins found in the root canal and makes them less toxic,and (d) it diffuses through dentinal tubules and may communicate with the periodontal ligament space to arrest external root resorption and accelerate healing [12, 13].

Despite its advantages, $Ca(OH)_2$ endodontic treatments failure in teeth has been reported. *Enterococcus faecalis* has been reported as the bacterial microorganism responsible for the failure of endodontic therapies [14]. *Enterococcus faecalis*colonizes inside dentinal tubules up to 300 microns, a site that is difficult to access for the action of $Ca(OH)_2$, affecting its effectiveness in endodontic therapy [15, 16]. Combining $Ca(OH)_2$ with an appropriate solvent may increase its solubility, antimicrobial activity, and access to complex root canal sites. The solvent used for $Ca(OH)_2$ influences both the physical and chemical properties of the material, including its viscosity and ion release pattern. A range of water-based materials has traditionally been used but there are alternative vehicles [17]. This study proposes using electrolyzed superoxidation solution (ESS) with neutral pH as the $Ca(OH)_2$ solvent for the endodontic treatment. ESS has a neutral pH (6.4–7.5) and is produced from the electrolysis of sodium chloride (NaCl) solution in an electrolytic cell where a diaphragm (partition or membrane) separates the anode and the cathode. Cations are drawn towards the electrode negative, where they receive electrons, forming a negatively charged antioxidant solution (alkaline solution). At the positive electrode, anions are attracted, which give up their additional electrons to create a positively charged oxidant solution (acid solution) [18]. For ESS formation, part of an antioxidant solution formed is channeled back into the anode chamber, thus increasing the content of hypochlorite ions (OCl^-).The reintroduction of the alkaline solution back into the acidic solution allows adjusting the pH up to neutral. Therefore, ESS is mainly composed of hypochlorous acid (HOCl), OCl^-, chlorine (Cl^-), and sodium hypochlorite (NaOCl). Electrolysis may produce H^+ and OH ions, H and OH radicals, H_2, O_2, HO_2, and O_3 due to redox reactions. As a result, hydrogen and ozone gas are released, and a percentage of hydroxides remains in the solution in various forms, including but not limited to hydrogen peroxide (H_2O_2) [19, 20].

ESS has become a new alternative in tissue asepsis in different areas of medicine and the food industry for its high antimicrobial activity, stability, and safety [21, 22]. Some reports suggest that the presence of Cl^- and a high concentration of oxidation-reduction potential (ORP) are responsible for the antimicrobial activity of ESS. However, other studies suggest that HOCl is the most active of compounds [23, 24].

A significant antimicrobial activity of ESS in removing biofilms from *E. faecalis* in root canals has been previously reported [25–29]. The aim of the present study was to evaluate the antimicrobial effect of combining $Ca(OH)_2$ with an ESS with neutral pH called OxOral®, in *E. faecalis*-infected root canals.

2. Materials and Methods

2.1. E. faecalis culture conditions. One cryovial containing *E. faecalis* (ATCC® 29212™) cells was rapidly thawed in a prewarmed 37 °C water, and cells were placed into 100 mL brain heart infusion broth (BHI; Oxoid Limited, Hampshire, UK) supplemented with 5 μg/mL hemin and 0.5 μg/mL menadione at 0.5 McFarland. The culture was incubated at 37 °C for 24 h under anaerobic conditions. Afterwards, 1 mL of the preculture of *E. faecalis* was collected, placed in 100 mL of BHI-supplemented broth, and incubated until reaching the exponential, logarithmic phase.

2.2. Instrumentation and Preparation of Teeth. Sixty human maxillary 2nd premolar teeth were obtained from human volunteers of the Endodontic Postgraduate Program of the School of Dentistry at the Autonomous University of Nuevo Leon. The study was conducted following the Declaration of Helsinki, the Institutional Ethics Committee approved the protocol (SPSI-01613/00249), and informed consent was obtained from each volunteer. The dental crowns were sectioned with a diamond disc and press. The working length was determined with a #15 dental file (K-Flex) of 25 mm; 1 mm was subtracted from its exit from the apical foramen. Instrumentation began with Triple-Flex Files Stainless Steel files (SybronEndo, Ormex, Yucatan, Mexico) up to file #40 using 5.2% NaOCl as an irrigating agent between each file change to maintain conduit permeability.

The apical region of each tooth was sealed with composite resin and externally with a layer of nail varnish, excluding the cervical opening, which was enlarged with Gates GLIDDEN files to be later autoclaved at 121°C for 15 min in 1.5 mL tubes. All root portions and materials used (cotton pellets, Eppendorf tubes, instruments, files, syringes, rotary drills, diamond disc, and serrated dissection forceps) were autoclaved.

2.3. Contamination and Treatment of Root Canals. Each root canal was inoculated with 15 μL of *E. faecalis* with a bacterial concentration of 5 $\log_{10}$ CFU/mL and incubated in an anaerobic atmosphere at 37 °C for 21 d to achieve the formation of *E. faecalis* biofilms within the root canal, after which 10 μl of supplemented BHI broth were added every two days to avoid teeth dehydration and promote bacterial growth.

After incubation, 12 samples were randomly selected and plated on BHI agar to verify contamination of the root canals by CFU counting.

Sixty root canals were randomly divided into four groups, and 10 μL of the treatment was applied within the root canal based on the following treatment groups: group 1, 0.9% NaCl; group 2, 0.9% NaCl plus $Ca(OH)_2$ paste; group 3, OxOral®; and group 4, OxOral® plus $Ca(OH)_2$ paste. $Ca(OH)_2$ medication pastes were prepared by mixing 0.5 g of $Ca(OH)_2$ powder plus three drops of saline solution (0.9% NaCl) or OxOral®. All treated teeth were incubated for up to 18 d.

2.4. Evaluation of Bacterial Growth. For *E. faecalis* growth evaluation, aliquots of 100 μL were taken at days 1, 6, 12, and 18 post-treatment, serially diluted, and plated onto BHI-supplemented agar using a glass spreader. Next, plates were incubated at 37 °C for 24 h, and CFU/mL were calculated; all measurements were performed in triplicate.

2.5. Measurement of pH Variation. For pH measurements, 60 tubes (15 mL) containing 2 mL of BHI-supplemented broth were inoculated with 10 μL of *E. faecalis* cells at ~5 $\log_{10}$ CFU/mL at 37 °C for 24 h. Tubes were then randomly divided into four groups and 9 mL of the different treatments were added. pH values were measured at days 1, 6, 12, and 18 post-treatment, by a digital pH meter (Corning®, Model 313; NY, USA) calibrated with buffer solutions (J.T.Baker-Avantor Performance Materials, S.A. de C.V, Mexico City, Mexico; pH = 4.00; pH = 7.00; and pH = 10.00) before each experiment. After removing the specimens, the container was placed in an orbital shaker (Prendo, INO-650 M, Mexico City, Mexico) for 5 sec before measuring. The room temperature during the test was 25 °C.

2.6. Statistical Analysis. Results were expressed as means ± standard deviation (SD) of the response of three replicate determinations per treatment from three independent experiments. The significance level was assessed by the ANOVA and Tukey's tests ($p < 0.05$), using the SPSS statistics software version 22.

3. Results

3.1. Evaluation of Antimicrobial Properties of OxOral® in the Root Canals. E. faecalis growth was determined at days 6, 12, and 18. One-way ANOVA test was performed, taking the bacterial growth ($\log_{10}$ CFU/mL) as the dependent variable and the treatment groups and the time as independent variables. The results show significant differences in the treatment groups and the time, with a $p = 0.001$ and $p = 0.012$, respectively.

Our results showed significant ($p < 0.05$) antimicrobial activity in the root canals treated with saline solution plus $Ca(OH)_2$, OxOral®, and OxOral® plus $Ca(OH)_2$ at 24 h with 0.051 ± 0.072, 0.044 ± 0.07, and 0.000032 ± 0.072 $\log_{10}$ CFU/mL, respectively. In addition, a sustained inhibitory effect was observed in saline solution plus $Ca(OH)_2$ and OxOral® until day 6 posttreatment. However, only the combination of OxOral® plus $Ca(OH)_2$ maintained its inhibitory potential against *E. faecalis* after 18 d post-treatment, as compared with the other treatments ($p < 0.05$), whereas saline solution showed a progressive increase in bacterial growth from 4.55 ± 0.071 log 10 CFU/mL (day 0 post-treatment) to 10.05 ± 0.075 (day 18 post-treatment), as expected(Table 1).

3.2. pH Measurements in the Treated Root Canals. The pH variation of the different treatments at days 0, 1, 6, 12, and 18 was also evaluated. One-way ANOVA test was performed, where the test variable was pH in function of the treatment groups and time. We observed a statistically significant ($p < 0.05$) difference only in the treatment group but no difference was observed with regard to time ($p > 0.05$).

pH measurements in the root canals treated with saline solution and OxOral® showed neutral pH maintenance from day 0 (baseline) with 6.25 ± 0.075 and 7.003 ± 0.321 until day 18 post-treatment with 7.41 ± 0.291 and 7.456 ± 0.196, respectively, whereas root canals treated with saline solution plus $Ca(OH)_2$ and OxOral® plus $Ca(OH)_2$ showed an alkaline pH from day 0 (baseline) with 12.149 ± 0.021 and 12.22 ± 0.033 until day 18 with 12.522 ± 0.02 and 12.586 ± 0.083, respectively. We did not observe statistical differences in pH values between these treatment groups (Table 2).

3.3. Consistency of OxOral® plus $Ca(OH)_2$ Combination. Figure 1 shows a comparison of the macroscopic characteristics of the combination of saline solution plus $Ca(OH)_2$ and OxOral® plus $Ca(OH)_2$, where we observe a higher solubility and pasty consistencies compared with the traditional combination.

4. Discussion

One of the requirements of an endodontic root canal sealer is that it should not be cytotoxic and immunologically compatible with peripheral tissue. Therefore, a specific root canal sealer biocompatibility and antimicrobial activity remain one of the major considerations for selecting an appropriate sealer for a dental restoration. $Ca(OH)_2$ has been widely used since the 1920s for its biocompatibility, antimicrobial potential, and ftissue restoration support in endodontic therapies [30].

Hydroxyl ions are responsible for $Ca(OH)_2$ antimicrobial activity.Therefore, the efficacy of any product varies according to the availability of these ions in solution, which in turn reflects the nature of the solvent used. Most existing commercial $Ca(OH)_2$ use vehicles of water. However, other agents have recently been mixed with water, such as glycerin or polyethylene glycol (PEG) [17].

The pH values from using OxOral are similar to some commercial $Ca(OH)_2$ products that use vehicles of water such as Calasept Plus™, Calcipulpe™, DT Temp™, Pulpdent™, and Ultracal XS™ with pH values between 11.8 and 12.7 [17, 31]. However, there are reports where the pH reaches up to 15.0 in $Ca(OH)_2$ products, where a mixture of water with PEG is used, such as Calmix™ [32].

Table 1: *Enterococcus faecalis* growth in the presence of 0.9% sodium chloride, 0.9% sodium chloride plus calcium hydroxide, OxOral, and OxOral plus calcium hydroxide.

Posttreatment time (days)	Treatment groups ($\log_{10}$ CFU/mL ± SD*)			
	0.9% NaCl	0.9% NaCl + $Ca(OH)_2$	OxOral	OxOral + $Ca(OH)_2$
0	4.55 ± 0.071	4.47 ± 0.07	4.6 ± 0.074	4.443 ± 0.068
1	8.02 ± 0.069	0.051 ± 0.072	0.044 ± 0.07	0.000032 ± 0.072
6	10.01 ± 0.07	0.1 ± 0.071	0.402 ± 0.071	0.0000031 ± 0.074
12	10.02 ± 0.073	1.201 ± 0.074	0.993 ± 0.07	0.00005 ± 0.074
18	10.05 ± 0.075	2.021 ± 0.071	1.904 ± 0.075	0.00003 ± 0.07

*The data represent mean ± standard deviation of $\log_{10}$ CFU/mL.

Table 2: pH values in the presence of 0.9% sodium chloride, 0.9% sodium chloride plus calcium hydroxide, OxOral, and OxOral plus calcium hydroxide.

Posttreatment time (days)	Treatment groups (mean pH ± SD*)			
	0.9% NaCl	0.9% NaCl + $Ca(OH)_2$	OxOral	OxOral + $Ca(OH)_2$
0	6.25 ± 0.075	12.149 ± 0.021	7.003 ± 0.321	12.22 ± 0.033
1	6.606 ± 0.092	12.268 ± 0.024	7.048 ± 0.4	12.448 ± 0.05
6	6.606 ± 0.092	12.268 ± 0.02	7.048 ± 0.429	12.448 ± 0.05
12	6.606 ± 0.09	11.794 ± 0.656	6.506 ± 0.495	12.272 ± 0.130
18	7.41 ± 0.291	12.522 ± 0.02	7.456 ± 0.196	12.586 ± 0.083

*The data represent mean ± standard deviation of pH measurements.

There are previous studies, where the use of neutral pH solutions is reported as a vehicle for the root canal $Ca(OH)_2$ pastes. For example, Tronstad et al. used a Ringer's solution (0.125 M NaCl, 1.5 mM $CaCl_2$ dihydrate, and 5 mM KCl; pH = 7.3 – 7.4) combined with $Ca(OH)_2$ and filled the root canal of green monkey teeth (*Cercopithecus aethiops sabaeus*), and they observed maintenance of alkalinity conditions with a pH = 12.2 after four weeks [33]. However, there are no reports on using ESS at neutral pH as a vehicle to prepare $Ca(OH)_2$.

Some studies suggest that $Ca(OH)_2$ is a slow-acting antimicrobial, which requires at least 24 hours to produce a bactericidal effect against *Enterococci* spp. Furthermore, $Ca(OH)_2$ hydroxide hydrolyzes the lipid moiety of bacterial LPS, causing its biological inactivation, the desired effect to prevent an inflammatory reaction in the periapical tissue [34, 35].

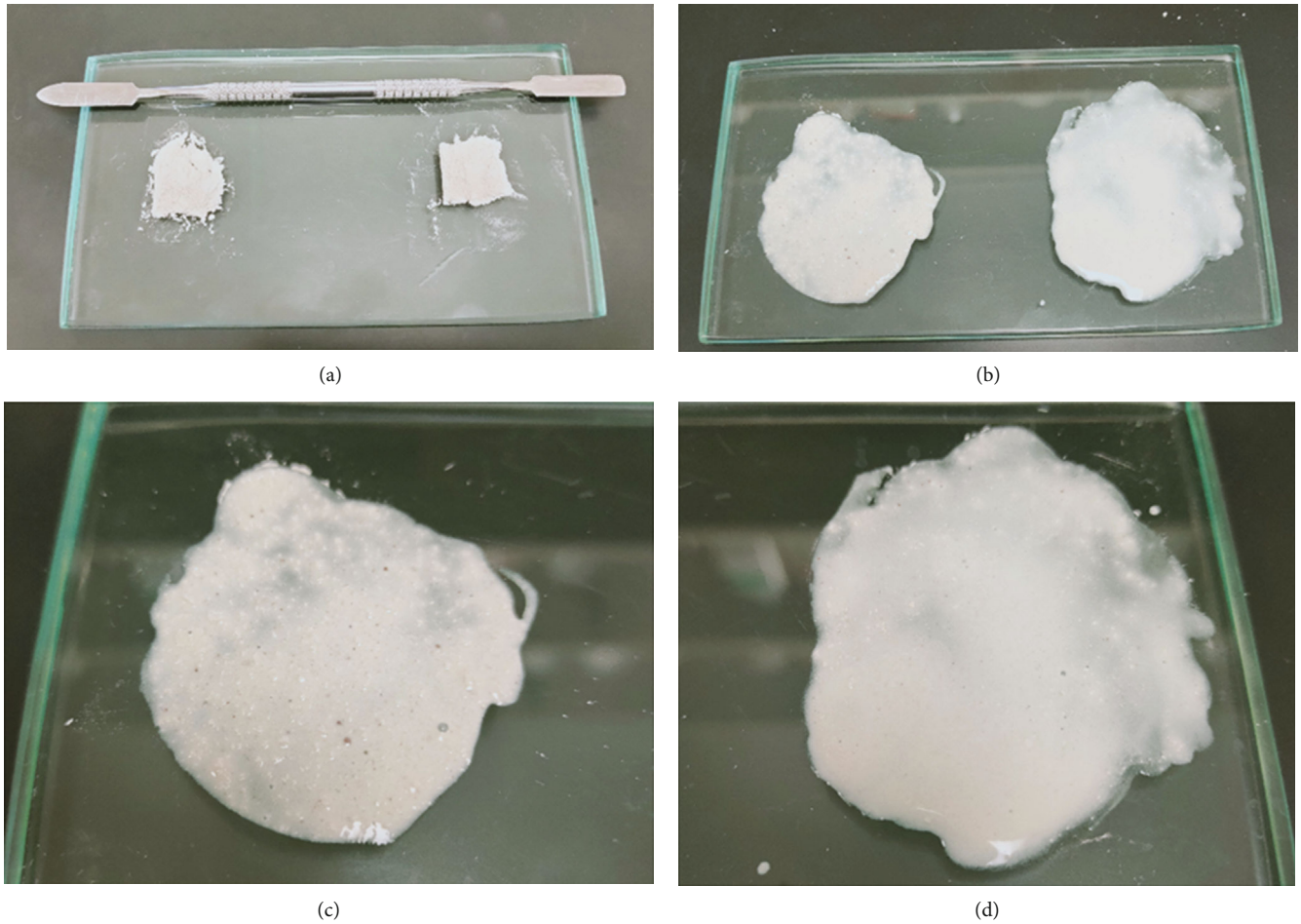
For many years, different compounds have been evaluated for root canal disinfection, $Ca(OH)_2$-based pastes being the most widely used.However, the permanence in time of these pastes is one of the main discrepancies between the authors, mainly in the cases of the teeth with pulp necrosis and periapical lesion [36, 37]. Our study observed the intracanal permanence of the $Ca(OH)_2$ plus saline solution or OxOral® pastes for up to 18 d.

Root treatment consists of debriding and disinfecting the entire root canal system, which requires eliminating the pulp tissue and the microorganisms causing the infection, adopting instrumentation and chemical irrigation mechanisms, and subsequently a medication in the root canal between treatment sessions. The success of endodontic treatment depends on the removal of microbes from the root canals and avoidance of reinfection.

Paudel et al. found that 0.9% NaCl was as effective as sequential use of 3% H_2O_2, 5.2% NaOCl, and 0.9% NaCl, although saline has no antibacterial activity [38]. In addition, it has been reported that the exposure and contact time during the irrigation of human root canals are crucial, since treatment with ozonized water, 2.5% NaOCl, 2% CHX, and gaseous ozone for 20 min was not sufficient to inactivate *E. faecalis* [39]. Therefore, in our study we evaluated the antimicrobial activity of treatments from one to 18 d posttreatment.

With a significant amount of calcium carbonate, the $Ca(OH)_2$ paste has a granular consistency due to its low solubility (1.73 g/L at 20 °C). One of the most interesting observations in the present study was the ease of removing the paste composed of $Ca(OH)_2$ plus OxOral®, unlike that composed of $Ca(OH)_2$ plus saline solution. However, other studies are necessary to evaluate any physical-chemical changes.

On the other hand, reactive oxygen and chlorine species are derived during the electrolysis process to produce electrolyzed water and have been previously evaluated in endodontic treatment. In this regard, Zhou et al. reported that a plasma jet with or without helium flowing through 3% hydrogen peroxide effectively sterilized root canals infected with *Enterococcus faecalis* [40]. Another study conducted by Mihadi et al. assessed the cytocompatibility and antibacterial activity of different concentrations of CHX combined with H_2O_2 compared with the action of 5.25% and 2.5%



(a) (b) (c) (d)

Figure 1: Comparison of the $Ca(OH)_2$ solvents. (a) Comparison of the amount of $Ca(OH)_2$ powder used; (b) comparison of the macroscopic characteristics of the combination of saline solution plus $Ca(OH)_2$ (left) and OxOral plus $Ca(OH)_2$ (right); (c) combination of saline solution plus $Ca(OH)_2$; (d) combination of OxOral plus $Ca(OH)_2$.

NaOCl. All combinations of CHX and H_2O_2 except 0.1% CHX plus 3% H_2O_2 were efficient irrigants against planktonic *E. faecalis* and had a better cytocompatibility with PDL cells than 5.25% and 2.5% NaOCl [41].

Some studies have reported a significant antimicrobial activity of electrolyzed superoxidized water [42, 43]. Different antimicrobial broad-spectrum disinfectants are manufactured by Esteripharma®, Mexico, SA of CV, each with different compositions and proposes. Velázquez-Meza et al. evaluated the antimicrobial activity based on superoxidized water called Estericide Qx® against 524 clinical bacterial isolates causing nosocomial infections, including Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa* beta-lactam resistant) and Gram-positive (*Staphylococcus aureus* and *Streptococcus epidermidis* methicillin-resistant, and *Enterococcus faecium*). The results showed that Estericide Qx® provides a broad-spectrum antibacterial activity mainly in Gram-negative [44]. Other studies, Lucio-Sauceda et al., reported a significant anti-*H. pylori* activity of OxOral® by microdilution assays, and Rivera-Garcia et al. observed a significant antimicrobial activity against *Listeria monocytogenes* on eggshells [18, 22].

Landa-Solis et al. treated pure cultures of *S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella typhi*, and *Candida albicans* with Microcyn® and found it was active on all microorganisms tested [45]. In addition, Vorobjeva et al. reported that superoxidized solution was effective on spores, Gram-positive, and Gram-negative bacteria related to nosocomial infections [46].

According to many studies, chlorine and a high concentration of ORP in ESS seem to be responsible for its antimicrobial activity. Active chlorine compounds destroys the membranes of microorganisms. Other modes of chlorine action (e.g., decarboxylation of amino acids, reactions with nucleic acids, and unbalanced metabolism after the destruction of crucial enzymes) also have been proposed [20, 47]. Studies suggest that HOCl is the most active of the chlorine compounds because HOCl penetrates cell membranes and produces hydroxyl radicals, which exert their antimicrobial activity through the oxidation of crucial metabolic systems. In addition, OH radicals, which are the most potent oxidizing agents, also have shown antimicrobial activity [24, 29, 48].

The main characteristics of $Ca(OH)_2$ are its limited solubility, high pH, and use as a broad-spectrum antimicrobial

agent. Despite the benefits and advantages of $Ca(OH)_2$, its use is cumbersome. Proper handling and placement are challenging for the dentist. In addition, its removal from the canal is usually incomplete, with 20-45% residue on the canal wall, even after copious irrigation using saline, sodium hypochlorite, or EDTA. Residual $Ca(OH)_2$may shorten the setting time of zinc oxide-eugenol endodontic sealants. In particular, it may interfere with the sealing of a root filling and affect the quality of the treatment.

On the other hand, the potential of $Ca(OH)_2$ to eliminate bacteria from the root canal has recently been challenged. Some *in vitro* studies have reported that dentin inactivates the antimicrobial activity of $Ca(OH)_2$, and a clinical study reported an increase in bacterial growth after its application. Our findings agree with these studies since the combination of $Ca(OH)_2$ plus saline solution showed an increase in bacterial growth of *E. faecalis* after day six of treatment. However, in the root canals treated with the combination of $Ca(OH)_2$ plus OxOral®, we did not observe *E. faecalis* growth. Therefore, it is possible that OxOral® may have a synergistic effect with $Ca(OH)_2$.

5. Conclusions

Taken together, the results of the present study demonstrated that the combination of OxOral® plus $Ca(OH)_2$ showed a significant antimicrobial activity and solubility of $Ca(OH)_2$, prolonging the alkaline pH conditions. Our results open the possibility for further research on the use of OxOral® for sealing root canals, during endodontic treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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