

Antimicrobial activity of ozone and NaF-chlorhexidine on early childhood caries

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Abstract: An early childhood carie (ECC) is an extremely destructive form of tooth decay. The aim of this study was to investigate the action of ozone (O₃), and the association of sodium fluoride (NaF) with chlorhexidine (CHX) on bacteria related to ECC. Overnight culture of the bacteria was performed. On exponential phase the suspension was adjusted (10¹-10⁸ CFU/mL). A drop (10µL) of each concentration of bacteria was applied on sheep blood agar plates and treated with O₃ (2, 20, 200, and 2,000 ppm); after 18 hours, recovery analysis of CFU verified the reduction of bacterial activity. For NaF-CHX, sterile 96-well plates were prepared and divided into groups: G1 (150 µL TSB); G2 (20 µL of bacteria + 25 µL CHX + 25 µL NaF); and G3 (150 µL TSB + 20 µL of bacteria + 50 µL water). The plates were verified by analysis of the optical density (0, 12, 14, 16, and 18 hours). The data from O₃ test were submitted to ANOVA and Tukey's test (p < 0.05). For the data from NaF-CHX, the ANOVA 2-way and Bonferroni's test (p < 0.05) were used. The number of CFU/mL showed death > 3log₁₀ (99.9%) for all bacteria (ozone ≥ 20ppm), while the combination of NaF-CHX was more effective (p < 0.001) compared to each substance tested alone and the control group. The antimicrobial agents tested were able to inhibit all bacteria tested; O₃ seemed to be a good alternative for controlling progression of carious lesions, while the association of NaF-CHX showed to be a good antimicrobial with easy and inexpensive application.

Keywords: Chlorhexidine; Dental Caries; Sodium Fluoride; Tooth Deciduous; Ozone.

Introduction

The American Academy of Pediatric Dentistry (AAPD) defines Early Childhood Caries (ECC) as the presence of one or more decayed deciduous teeth, missing (due to caries) or restored before 71 months of age. Studies indicate large quantities of *S. mutans* and *Lactobacillus* in dental caries of deciduous teeth¹. Restorative therapy in primary teeth exposed to ECC is essential, but not always possible. It is relatively common for children to present resistant behavior, especially at an early age.

Partial caries removal, based on the philosophy of minimal intervention, may be the treatment of choice in some cases. Unfortunately, in some children, the caries lesions progress to a point that treatment under general



anesthesia is required if there is no collaboration of the child.² Other limiting factors are the size and shape of the ECC's lesion, which often do not allow the adaptation of a suitable restorative material.² Therefore, there is a need to find a way to immediately treat those lesions and consequently inhibit or halt the caries lesion in these patients until the appropriate behavior and cooperation are achieved. Materials available for this purpose include fluoride and chlorhexidine, and the use of ozone gas (O₃) has also been recently reported.

The use of fluoride in its various forms is a major contributor to the decrease in the prevalence of caries worldwide, and also reduces the severity and progression of lesions. Fluoride has three main mechanisms of action: amendment of bacterial metabolism after diffusion into the interior of the bacteria in the form of hydrofluoric acid (HF), inhibition of demineralization, and aid in remineralization.³

Chlorhexidine is the gold standard anti plaque agent. Its ability to bind to soft and hard tissues in the oral cavity enables it to act for a long period after application. It can be bacteriostatic or bactericidal depending on the dose. It acts against a wide array of bacteria including Gram positive and Gram negative.⁴

Ozone gas (O₃) has been tested in restorative dentistry and endodontics.⁵ Topical administration can be performed in gaseous form through an open system or through a suction sealing, as a prerequisite to avoid inhalation and adverse effects. The action of O₃ is related to its reacting capacity with lipid double bonds, thus leading to bacterial wall lysis and bacterial cell content extravasation. By entering the cell, O₃ promotes oxidation of nucleic and amino acids; and cell lysis depends on the extent of these reactions.⁶ Several questions about the effect of ozone remain unclear, for example: the ideal ozone concentration; delivery forms; and the ideal time to reach full antimicrobial efficacy. Studies have shown controversial results on *S. mutans* and *L. casei*,^{7,8} and negative results on *E. faecalis*.^{5,9} Therefore, despite the fact that *E. faecalis* is not related to ECC, in order to test the efficacy of O₃, the technique should be tested in bacteria considered more vulnerable and more resistant.

Due to the high prevalence of ECC in the world population and the difficulty of behavior of many patients facing the immediate restorative treatment, there is a need to find a treatment option that is effective in controlling the progression of caries lesions in deciduous teeth. Thus, we intend to investigate an antimicrobial agent that can be used clinically in pediatric patients, and is easily applied and accessible to the dentist. This study aims to test the hypothesis that ozone, and the association between chlorhexidine and NaF, will have positive results in antimicrobial action in bacteria related to early decay.

Methodology

Bacterial growth condition

The antimicrobial activity of agents was tested against standard strains of microorganisms. Specimens of *Streptococcus mutans* (ATCC 10449 serotype c), *Lactobacillus acidophilus* (ATCC 4356), and *Enterococci faecalis* (V583) were used. The stock cultures were stored in skim milk at -80°C. Inoculum from those stock cultures were cultivated in Wilkins-Chalgren Anaerobe broth (Oxoid Ltd., Basingstoke, Hampshire, UK) in an atmosphere of 5% CO₂/10% H₂/85% N₂ (Coy anaerobic chamber, Coy Laboratory Products Inc., Ann Arbor, MI, USA) after being screened by Gram-staining to confirm purity. The bacteria were also cultivated on sheep blood agar plates for the analysis of colony morphology and confirmation of purity. Loopful inoculations of *S. mutans*, *L. acidophilus*, and *E. faecalis* were transferred to 10 mL of appropriate broth and incubated at 37°C under anaerobic conditions. Each microbial strain suspension was adjusted to the turbidity level, corresponding to tube #1 of the McFarland scale, for an approximate concentration of 3 × 10⁸ cells per mL.

Effect of ozone on *S. mutans*, *L. acidophilus*, and *E. faecalis*

We used two ozone generators with different gas production capacity: OL80A (2 and 20 ppm) and OL80W (200 e 2,000 ppm), both fabricated by Yanco Industries LTD, USA. These devices produce O₃ inside a chamber, where it is capable of reaching concentrations from 2 to 2,000 ppm.

An overnight culture (early exponential phase) of all three bacteria was adjusted in suspension of 10^8 CFU/mL (#1 MacFarland Standard), followed by log dilutions ranging in concentrations of 10^8 - 10^0 CFU/ml.

Before the treatment with ozone, we applied 10 μ L of each concentration of bacteria as droplets on the surface of sheep blood agar (Trypticase™ Soy Agar with 5% Sheep Blood) plates. Six plates were prepared as described in Table 1. Then, each plate was placed separately into the chambers according to the ozone concentration to be tested. The exposure time in this experiment was 4 minutes, based on results obtained in a previous study.¹⁰

The plates were incubated in an anaerobic chamber (10% H₂ - 5% CO₂ - 85% N₂) at 37°C for 18 hours. After this period, the number of colonies tested in different concentrations was calculated using positive control (no treatment) as the standard plate. The analyses were performed in triplicate.

Effect of sodium fluoride (NaF) and chlorhexidine (CHX) on *S. mutans*, *L. acidophilus*, and *E. faecalis*

The substances tested were 0.12% CHX (Peridex®, 3M, WA, USA) and 5% NaF (JT Baker, Center Valley, PA, USA). In previous tests, we observed that concentrations of the substances mentioned above were able to inhibit bacterial growth completely under the conditions tested. Therefore, we decided to dilute the concentrations to the point that there was no inhibition of bacterial growth. Thus, we chose to test the substances at the following concentrations: NaF (5/ 2.5/ 1.25/ 0.625/ 0.31/ 0.15/ 0.07/ 0.03) and CHX (0.0004/ 0.0002/ 0.0001/ 0.00005/ 0.000025/ 0.000012/ 0.000006). All concentrations of these substances were

Table 1. Description of each plate.

Plate	Inoculum	O ₃ concentration
1- Negative control	No	No treatment
2- Positive control	Yes	No treatment
3- Experimental	Yes	2 ppm
4- Experimental	Yes	20 ppm
5- Experimental	Yes	200 ppm
6- Experimental	Yes	2,000 ppm

tested separately and together using the mixing method known as Checkboard.¹¹

Sterile 96-well plates were prepared so that each well contained a final volume of 220 μ L. The columns were distributed in numbers 1-8 (NaF) and the lines in letters "A" through "G" (CHX). The compositions of the wells were:

- Experimental wells: 150 μ L TSB, 20 μ L of each bacterium 10^6 UFC/mL, 25 μ L CHX, and 25 μ L NaF.
- Bacteria control: 150 μ L TSB, 20 μ L of each bacterium 10^6 UFC/mL, 50 μ L sterile distilled water.
- Medium control (TSB): 150 μ L TSB + 70 μ L sterile distilled water.

After the preparation of microplates, the optical density (λ 610 nM) of the samples was verified (T=0 hours), using the Vmax kinetic microplate reader/ SoftMaz Pro 3.1 (Sunnyvale, California, United States). Analyses of optical density were performed on the following times: 12 hours, 14 hours, 16 hours, and 18 hours. We kept the plates in the chamber at 37°C throughout the experiment period. A single operator performed this study in triplicate.

Statistical analysis

To analyze the action of ozone, the mean and standard deviation of the data were calculated and then the Analysis of Variance (ANOVA) was applied. There was statistical difference between the means, so Tukey's test was used to evaluate statistical differences for each of the criteria and their interactions.

For the experiments with CHX and NaF, the data was submitted to analysis of variance of two factors (two-way ANOVA). Then, Bonferroni correction for multiple comparisons was performed. In both tests, statistical analyzes were blinded to the type of bacteria. Analyses were performed by the Statistical Package for Social Sciences (SPSS, version 20.0, USA).

Results

Antimicrobial activity of ozone

Figures 1, 2, and 3 show the inhibition of bacterial growth on a logarithmic scale log₁₀ after treatment at different concentrations of O₃. Data shows a similar

pattern of inhibition for all three bacteria tested. *L. acidophilus* (Figure 2) and *E. faecalis* (Figure 3), when subjected to application of 2ppm of ozone, presented a slight resistance of these microorganisms to the gas. At concentrations ≥ 20 ppm, the inhibition was greater than $3\log_{10}$ (99.9% kill) ($p < 0.01$) for all three bacteria.

Antimicrobial activity of NaF-CHX

Table 2 shows the effect of each antibiotic in each bacteria compared to their control group (optical density “baseline” for each bacterial species). All three antibiotics tested on *S. mutans* acted better when compared to other microorganisms. Comparing *E. faecalis* and *L. acidophilus*, there was a weak tendency

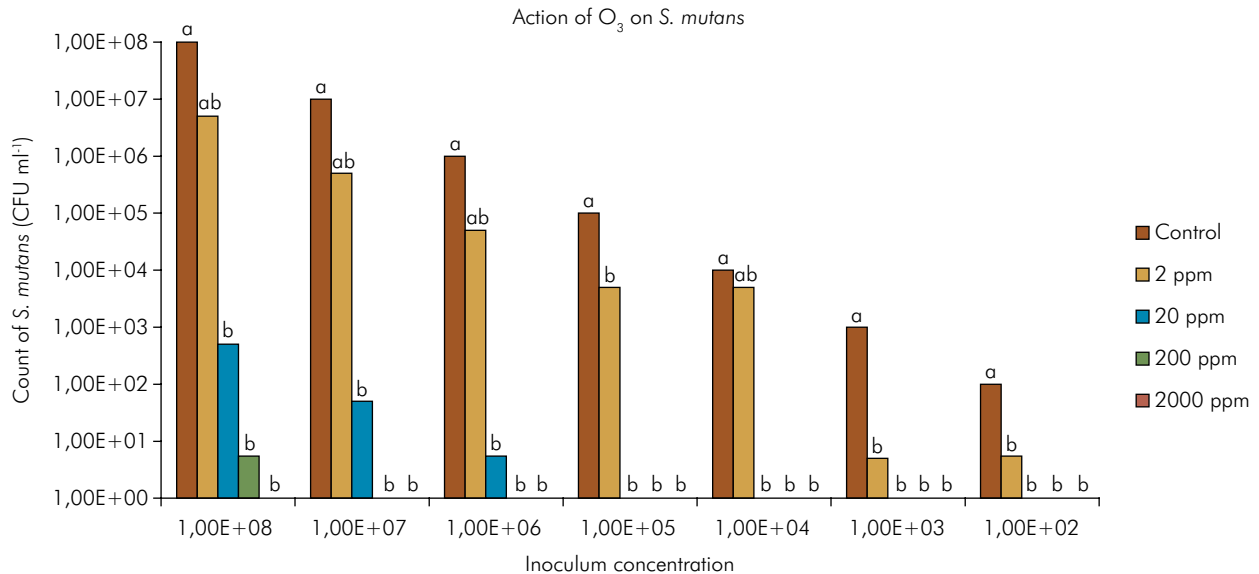


Figure 1. Inhibition of *S. mutans* growth in logarithmic scale (\log_{10}) after O_3 treatment. Logarithm values followed by the same lower case letters (for comparisons between O_3 concentrations) do not differ by Tukey’s test (significance level of 5%).

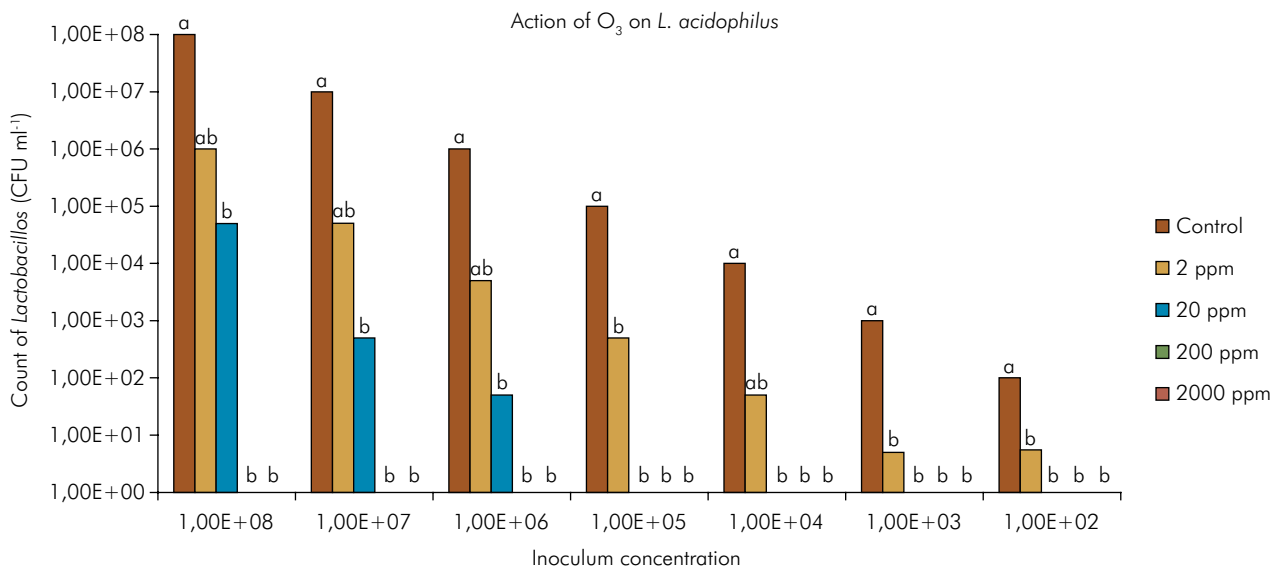


Figure 2. Inhibition of *L. acidophilus* growth in logarithmic scale (\log_{10}) after O_3 treatment. Logarithm values followed by the same lower case letters (for comparisons between O_3 concentrations) do not differ by Tukey’s test (significance level of 5%).

to see a more favorable effect of *L.acidophilus* when undergoing treatment with CHX and NaF separately; nonetheless, there was no difference for the NaF-CHX.

Figure 4 shows the analysis of the effect of each antibiotic and combination (NaF-CHX), by type of bacteria. The two substances were effective in controlling bacterial growth for the three bacteria tested when compared to the control group ($p < 0.001$). On *E.faecalis* there was no difference between CHX and Control, NaF and NaF-CHX were better than control or CHX, and there was no difference between NaF-CHX or NaF. In the test for *L.acidophilus*, all antibiotics were

better than control: NaF-CHX was better than any of the substance tested alone, and there was no difference between CHX or NaF. For *S. mutans*, NaF-CHX was more effective than any other substance tested alone and the Control group, NaF was better than CHX, and CHX was better than Control group.

Discussion

This study was developed with the purpose of identifying an antimicrobial agent able to inhibit or halt ECC caries until the appropriate behavior and

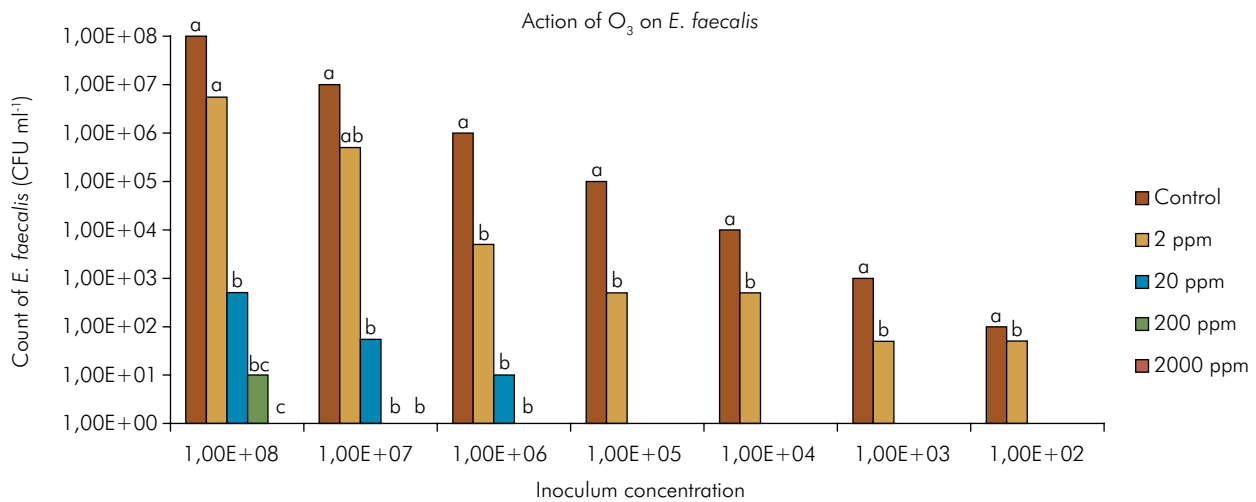
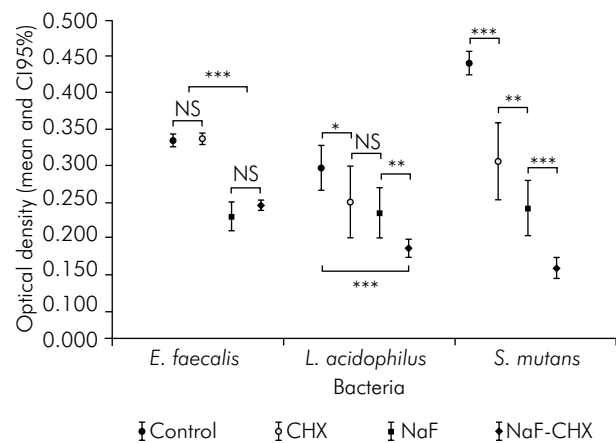


Figure 3. Inhibition of *E. faecalis* growth in logarithmic scale (\log_{10}) after O_3 treatment. Logarithm values followed by the same lower case letters (for comparisons between O_3 concentrations) do not differ by Tukey’s test (significance level of 5%).

Table 2. The effect of antimicrobials in the optical density of bacterial growth

Antimicrobials	Bacteria	Mean difference of optical density compared to control	p-value
CHX	<i>E. faecalis</i>	0.002	0.089 ^a
	<i>L. acidophilus</i>	- 0.049	0.028 ^b
	<i>S. mutans</i>	- 0.138	< 0.001 ^c
NaF	<i>E. faecalis</i>	- 0.110	0.088 ^a
	<i>L. acidophilus</i>	- 0.065	< 0.001 ^b
	<i>S. mutans</i>	- 0.206	< 0.001 ^c
CHX + NaF	<i>E. faecalis</i>	- 0.093	0.326 ^a
	<i>L. acidophilus</i>	- 0.115	< 0.001 ^b
	<i>S. mutans</i>	- 0.292	< 0.001 ^c

CHX: Chlorhexidine; NaF: Sodium fluoride. The p-values refer to the test of interaction between the effect of the antibiotic and the bacteria compared the following groups: a: *E. faecalis* and *L. acidophilus*; b: *L. acidophilus* and *S.mutans*; c: *E. faecalis* and *S.mutans*.



CI: Confidence Interval; NS: No significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 4. Effect of CHX, NaF and NaF-CHX on *S. mutans*, *L. acidophilus* and *E. faecalis*.

cooperation of the patient are achieved, allowing for routine dental care. The results showed that the hypothesis was accepted, since all the substances tested showed antimicrobial activity. Ozone is considered a strong oxidizer of the cell walls and cytoplasmic membranes of bacteria and is considered a potent bactericidal, antiviral, and antifungal agent.¹² It is important to point out that this statement is based on the use of ozone in blood, not gassing ozone to a microfilm.^{12,13} This is a common finding in the literature, extrapolating the use and effect of systemic ozone dissolving it in the bloodstream, to the dental field.^{12,14} These articles explain the indications and mechanism of action of ozone dissolved in blood but there is no reference to the effect in the oral cavity. Therefore, the real ability of ozone to kill cariogenic bacteria is yet to be determined.

From the three bacterial strains employed in this study, two were selected to represent pathogenic bacteria commonly present in ECC (*S. mutans* and *L. acidophilus*), and *E. faecalis* was known to be more resistant to antibiotics^{5,9}. Our results showed that the application of O₃ completely prevented the *in vitro* growth of all bacterial strains.

The use of agar-sheep-blood for bacterial culture in Petri dishes is a usual practice in microbiology, including the evaluation of bactericidal effects of different substances, such as ozone.^{15,16} Using similar methodology, an *in vitro* study revealed a reduction of viability of *E. coli*, *S. aureus*, and *Listeria innocua* under application of O₃ (2ppm / 4 hours).¹⁶ Another study showed that a mixture of 20 mg of O₃/mL + O₂ (1% O₃/99%O₂) in a single application for 5 minutes is able to effectively inhibit bacterial growth of *E. faecalis*, *Staphylococcus aureus*, and *Escherichia coli*.¹⁵ In all the studies cited above, the bacteria were cultured on agar in Petri dishes as well as in other culture media, and both studies considered agar as the best culture media for measuring the efficacy of O₃. This study showed success in applying O₃ for 4 minutes in all three bacteria tested. It is pertinent to emphasize that both generators (OL80A and OL80W) produce gas within chambers, and to achieve the desired final concentration takes about 20 minutes, adding 4 more minutes in target concentration. Polydorou et al.¹⁷ showed bactericidal effects of O₃ on *S. mutans* after

the application for 80 seconds, and suggested other studies on different bacteria. Estrela et al.⁵, reported satisfactory results on inhibiting growth of *S. mutans*, *L. casei*, and *A. naeslundii* using ozonated water, although the authors reported a limitation of delivering ozone gas from the equipment used. Polydorou et al.¹⁸, found complete inhibition of *S. mutans* growth after a 1-minute application even after a follow up of 8 weeks; however, the method showed limited effect on *L. casei*. Hems et al.¹⁰, reported limitation using O₃ gas. However, they showed a positive effect on planktonic *E. faecalis* with ozonated water after 4 minutes. As in the present study, most of the studies tested ozone generator for laboratory use only, we believe there is still a need to find equipment for clinical use.

For the method that assesses bacterial death to be considered efficient, it is expected to decrease by at least 3log₁₀ (99.9%) in the number of bacteria detected¹⁹. Using this parameter and standardizing the initial concentration of the three bacteria, we observed a logarithmic ≥ 3log₁₀ kill at all conditions tested, except for *L. acidophilus* and *E. faecalis* when subjected to application of 2 ppm of ozone. This suggests a slight resistance of these microorganisms to the gas, but this does not alter the results, since the equipment available and the methods that have been used and proven in the literature used a concentration above 2,000 ppm of ozone.^{7,10,17,18} Under the lab condition tested in this study, the results show complete inhibition of all the three bacteria using the concentration ≥ 20 ppm. We suggest more studies in order to test the efficacy of O₃ under clinical situations and with lower doses of the gas. The present data show antimicrobial O₃ effect on *E. faecalis*. This is in agreement with another study that found similar results testing ozone in the liquid (ozone bubbles), which demonstrated that the solution has a bactericidal effect on *E. faecalis* in planktonic surface, and suspended in liquid.¹⁰ Nagayoshi et al.,²⁰ also showed the sensitivity of *E. faecalis* to ozone, which the authors examined *ex vivo*, the effect of ozonated water on *E. faecalis* and *S. mutans*, and also verified cytotoxicity in mouse fibroblasts. They concluded that ozonated water had a favorable effect and that has low cytotoxicity. Even though *E. faecalis* is not related to ECC, it was selected for this study because it has been described in the literature as more resistant to the

action of antimicrobial agents.^{5,10} The main difference between our study and others is the ozone generator used and the method of application.

Both gaseous and aqueous ozone have been reported to exert antimicrobial effects.^{10,21} The aqueous form of ozone is a potential antiseptic agent and shows less cytotoxicity than gaseous ozone.²² Overwhelming evidence shows that the bronchial-pulmonary system is very sensitive to ozone and that this gas should never be inhaled. The respiratory tract lining fluid is constituted of a very thin, watery film containing a minimal amount of antioxidants that makes mucosal cells extremely vulnerable to oxidation.²³ Known side effects are epiphora and upper respiratory irritation, rhinitis, cough, headache, occasional nausea, and vomiting.²⁴ Cytotoxicity is less relevant when applying ozone gas onto carious tooth hard substance via a sealing suction system as a prerequisite to avoid inhalation.²²

Ozone generating equipment converts oxygen to ozone. It is delivered through a system composed of a hand piece fitted with a silicone cup. This ensures close contact between the silicone cup and the carious area of the tooth so that the ozone does not escape. In previous tests with commercialized ozone generators, it was not possible to achieve the ideal sealing enabling the creation of an impermeable layer or the so called vacuum effect. Therefore, we decided to use a device that produced O₃ in chambers. So, from the results found in this study, we suggest that there is a need for improvement of equipment for clinical use, particularly with silicone tips that fit onto deciduous teeth.

The data from this investigation, together with other *in vitro* studies cited here, indicate that the use of ozone gas may be an alternative for infections caused by *S. mutans*, *L. acidophilus*, and *E. faecalis*. However, there is a need for other tests in bacterial biofilms and clinical studies with proper controls and adequate sample size for the validation of this technique.

The antimicrobial activity of NaF-CHX on planktonic species of oral pathogens was evaluated by minimum inhibitory concentration (MIC) assays. Although NaF and CHX exhibited antimicrobial activity against all bacteria tested, MIC results revealed that the association of NaF + CHX is more effective

than agents tested alone. Although both substances presented antimicrobial activity alone in all tested microorganisms, it is not feasible to compare the effect of NaF versus CHX.

Chlorhexidine (CHX) is one of the most widely used oral antimicrobial agents and is available in different formulations.²⁵ The substance is known to have good substantivity and at high concentrations (0.12% or more) is bactericidal, causing a lethal damage to the bacterial membrane, being active on both gram-negative and gram-positive bacteria.⁴ The CHX as antiplaque and anti-gingivitis agent remains as the gold standard, but its use as an anti-carries agent particularly in established lesions has been considered controversial, based on inconclusive clinical findings.²⁶ In the presence of SLS (sodium lauryl sulfate), which is the most commonly used surfactant in dentifrices, CHX has its efficiency decreased due to cationic (CHX) and anionic (SLS) reactions.²⁷ As the purpose of the study is to propose a substance that is effective in halting the ECC, the tested solution (NaF-CHX) was formulated starting from compounds in its purest form available on the market, without adding any other substance. Under the test condition, our results are similar to those found in the literature, proving its effectiveness in reducing the number of *S. mutans*^{28,29} on *L. acidophilus*³⁰ and on *E. Faecalis*.³¹

In this study, fluoride was used in the form of sodium fluoride (NaF), and under the conditions tested, NaF had a satisfactory antimicrobial effect on all bacteria tested. There are reports of direct antimicrobial activity against cariogenic bacteria.³² In the presence of low extracellular pH, the F⁻ is transported as hydrofluoric acid (HF) into the bacterial cell, where it then dissociates into H⁺ and F⁻.³³ The excess acidification of the cytoplasm may also inhibit the glucose transport mechanism inside the cell. Although these mechanisms have been demonstrated in cell culture, there is no proof of an antimicrobial effect of fluoride clinically. It is well documented that fluoride has the ability to inhibit or even reverse the initiation and progression of dental caries.^{34,35,36} Therefore, in clinical situations where there is a need for an urgent and effective treatment for halting the ECC, the benefits of using NaF and CHX seem to be a good alternative.

The data obtained in this study suggest that there is an additive effect when there is a combination of NaF and CHX. This is clearly demonstrated by the fact that under the conditions of the study, both substances showed antimicrobial effects alone, but showed better results when used in combination. This finding corroborates with the findings reported by other authors,³⁷ testing *in vitro* the combination of NaF-CHX on *S. mutans* and *S. sobrinus*, and found significant increases in the antimicrobial effectiveness of the solutions. The benefit of the association is attributed to the low molecular weight of the fluoride ion, which allows it to reach niches inaccessible to CHX, as in the case of incipient caries.³⁸ Furthermore, extremely high concentrations of CHX did not seem necessary for a lasting antimicrobial effect on bacterial colonization on root surfaces.³⁹ Therefore, with the exception of a limited number of pathogens such as *S. mutans*, *L. acidophilus*, and *E. faecalis*, most indigenous oral microorganisms are benign or beneficial. Therefore, the use of high concentrations of any antimicrobial substance can be detrimental to the host.⁴⁰

The bactericidal effect of NaF-CHX in *S. mutans* and *L. acidophilus* demonstrated in this study reinforces the advantage of combining these two substances for controlling the progression of dental caries. In general, the idea of associating NaF-CHX aims to gain control of bacterial proliferation (CHX) associated with reversing

the established caries (NaF). Therefore, this study demonstrated that the combination of antimicrobials with different antimicrobial mechanisms allows for a more effective treatment strategy against pathogens related to severe childhood caries.

Conclusion

The antimicrobial agents tested were able to inhibit *S. mutans*, *L. acidophilus*, and *E. faecalis*. The technique of using ozone gas seems to be a good alternative for controlling the progression of carious lesions in children. However, there remains a need to develop a generator suitable for clinical use. The association of NaF-CHX was shown to be a good antimicrobial agent with an easy application method and faster clinical applicability. Further investigation should be performed to confirm these results and to develop protocols for the use of such products to prevent the progression of severe childhood caries.

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