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ORIGINAL ARTICLE

Antibacterial Effect of Hypochlorous Acid on Bacteria Associated with the Formation of Periodontal Biofilms: An in vitro Pilot Study

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ABSTRACT

Objective: To evaluate the antibacterial effect of electrolytically generated hypochlorous acid on *Streptococcus gordonii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*. **Material and Methods:** In this in vitro experiment, the effect of hypochlorous acid (HOCl) on the strains *S. gordonii*, *F. nucleatum*, and *P. gingivalis* was evaluated using 4% sodium hypochlorite, 0.12% chlorhexidine, and distilled water as controls. The four groups were placed on each plate, and each group was replicated five times. The agar diffusion method by zones measurement was used. The data were processed with SPSS using the Kruskal-Wallis test and multiple comparison tests. **Results:** Hypochlorous acid showed an average inhibition halo of 9.28 mm on *S. gordonii*. As expected with distilled water, no zone of inhibition was noted for any of the bacteria, nor were zones of inhibition observed with HOCl for *F. nucleatum* and *P. gingivalis*. **Conclusion:** Hypochlorous acid showed antimicrobial properties against only *S. gordonii* and was less effective than 4% sodium hypochlorite and 0.12% chlorhexidine, although no significant differences were found between the latter.

Keywords: Microbiology; Anti-Bacterial Agents; Hypochlorous Acid; Sodium Hypochlorite.

Introduction

The control of microorganisms and biofilms is one of the measures used to prevent common oral diseases, such as caries and periodontitis, which can only be achieved by mechanical methods such as tooth brushing and flossing. Mouthwashes are widely used and have relatively complicated formulas, and most of them contain antimicrobial agents such as chlorhexidine (CHX), triclosan, cetylpyridinium chloride, chlorine dioxide, and cationic peptides [1,2]. Although these substances have marked antibacterial effects, oral rinses that do not alter the normal oral ecosystem but can significantly reduce biofilms are preferred for daily use.

Periodontal disease is mainly associated with the formation of bacterial biofilms. The main periodontal pathogens are gram-negative and anaerobic bacteria, some of which are highly proteolytic and cause bad breath [3]. Although selective antibacterial agents against these bacteria are not available, an overall reduction in the number of these bacteria will contribute to the control of periodontal disease [4].

Although the role of *S. gordonii* in the formation of subgingival biofilms is not defined, it has been shown in in vitro studies that when *P. gingivalis* depends on signals produced by *S. gordonii* to form mixed biofilms with *P. gingivalis* [4].

Hypochlorous acid is the active component of sodium hypochlorite without its adverse effects; thus, it could be considered a potent antiplaque for use in oral cavity as it has been shown to have a high antimicrobial effect [5]. HOCl has been shown to have a broad-spectrum antimicrobial effect at concentrations ranging from 0.1 to 2.8 mg/ml over a 2-minute exposure period. This microbicidal activity, although more effective for bacterial forms than spores and fungi, encompasses clinically relevant microorganisms such as Gram-negative and Gram-positive bacteria, parasites, and fungi [6].

In recent years, interest has increased in new high-potency molecules with antiplaque effects and bioequivalence with CHX but fewer adverse effects. Hypochlorous acid (HOCl) has been proposed as an antiplaque agent and as an agent for the healing of wounds in the oral mucosa due to its low toxicity, proven antimicrobial effectiveness, anti-inflammatory and cell proliferation-inducing effects, and history of use as a topical substance for wound disinfection in medicine [7]. Regulation of the normal flora contributes to periodontal health, and HOCl appears to have the ability to attack gram-negative pathogens during periodontitis [8]. However, the lack of studies necessitates further investigation of the effect of HOCl on oral microorganisms, especially those that form biofilms associated with highly prevalent diseases such as periodontitis.

The objective of this study was to evaluate the antibacterial effect of electrolytically generated HOCl ('electrolyzed water') on three of the main microorganisms associated with the formation of periodontal biofilms: *Streptococcus gordonii* ATCC 51656, *Fusobacterium nucleatum* ATCC 10953, and *Porphyromonas gingivalis* ATCC 33277.

Material and Methods

Study Design

This research was an in vitro experimental study carried out in the Bacteriology Laboratory of the College of Sciences at the Universidad Peruana Cayetano Heredia (Cayetano Heredia University), Lima, Peru.

200 ppm Hypochlorous Acid Preparation

To obtain the HOCl, the EcoloxTech 240 System (EWCO, Miami Beach, FL, USA) was used. One liter of distilled water and 1 g of sodium chloride plus acetic acid were added (calibration at pH 0.7), which yielded 200 ppm chlorine in HOCl. [9].

Strains Used

The strains used were *S. gordonii* ATCC 51656, *F. nucleatum* ATCC 10953, and *P. gingivalis* ATCC 33277.

Antibacterial Susceptibility Tests

To evaluate antibacterial effects, plates containing brain heart infusion (BHI) agar for *S. gordonii*, BHI supplemented with 5% sheep blood plus menadione and vitamin K for *F. nucleatum*, and BHI agar supplemented with horse blood plus menadione and vitamin K for *P. gingivalis* were monitored for 24 hours to confirm sterility [10,11].

The strains were cultured in BHI broth for 24 hours, and then, the turbidity was calibrated to 0.5 on the McFarland scale by using a swab to soak up the previously prepared inoculum and then streaking the surface of the agar four times. Next, the agar was allowed to rest for 5 minutes, and then, 6-mm filter paper discs (Whatman 3, Danaher Corporation, Washington, D.C., USA) impregnated with 10 µl of 200 ppm HOCl, 10 µl of 4% NaClO, 10 µl of 0.12% CHX, and 10 µl of distilled water were placed on the plate. Then, all plates were incubated at 37 °C for 48 hours under anaerobic conditions [11]. This procedure was repeated five times.

Four groups were formed: HOCl, 4% sodium hypochlorite (NaClO), 0.12% CHX, and distilled water. After 48 hours of incubation, the plates were examined, and the zones of inhibition were measured in millimeters using a calibrated Truper caliper. The four groups were placed on each plate, and each group was replicated five times. The number of plates to use (five) was determined assuming a maximum difference of 1.2 mm between the treatment means and a common standard deviation of 0.5 mm, with a type I error of 5% and power of 80%, necessary for the analysis of variance using Minitab 19 (Minitab LCC., State College, PA, USA).

Statistical Analysis

The data were processed with SPSS version 26 (IBM Corp., Armonk, NY, USA) using the nonparametric Kruskal-Wallis test and multiple comparison tests to compare the antimicrobial susceptibility to HOCl, including three controls, based on the diameter ranges of the zones of inhibition. The Kruskal-Wallis test was adopted as an alternative to the analysis of variance due to the evident non-normality and heterogeneity of the errors in analysis of variance. A p-value of 0.05 was considered statistically significant.

Results

Measurement of the zones of inhibition in the five plates at 48 hours of incubation allowed us to perform the comparisons provided in Table 1 for each of the oral bacteria under study.

Table 1. Antimicrobial effect of hypochlorous acid on *S. gordonii*, *F. nucleatum*, and *P. gingivalis*.

Acid	*S. gordonii* Mean ± SD	*F. nucleatum* Mean ± SD	*P. gingivalis* Mean ± SD
Hypochlorous Acid	9.28 ± 0.32^{ab}	0 ± 0^{a}	0 ± 0^{a}
4% Sodium Hypochlorite (NaClO)	11.74 ± 0.59^{bc}	11.06 ± 0.80^{b}	11.18 ± 0.50^{b}
0.12% Chlorhexidine	15.20 ± 0.66^{c}	12.64 ± 0.45^{b}	15.52 ± 0.43^{b}
Distilled Water	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
ANOVA: P-value	0.000	0.000	0.000
Levene test for homogeneity of variances: P-value	0.032	0.008	0.000
Kruskal-Wallis, multiple comparisons$^{(a,b,c)}$	0.000	0.000	0.000

HOCl showed an effect on *S. gordonii* (Figure 1A). As expected with distilled water, no zone of inhibition was noted for any of the bacteria, nor were zones of inhibition observed with HOCl for *F. nucleatum* ATCC (Figure 1B) 10953 and *P. gingivalis* ATCC (Figure 1C).

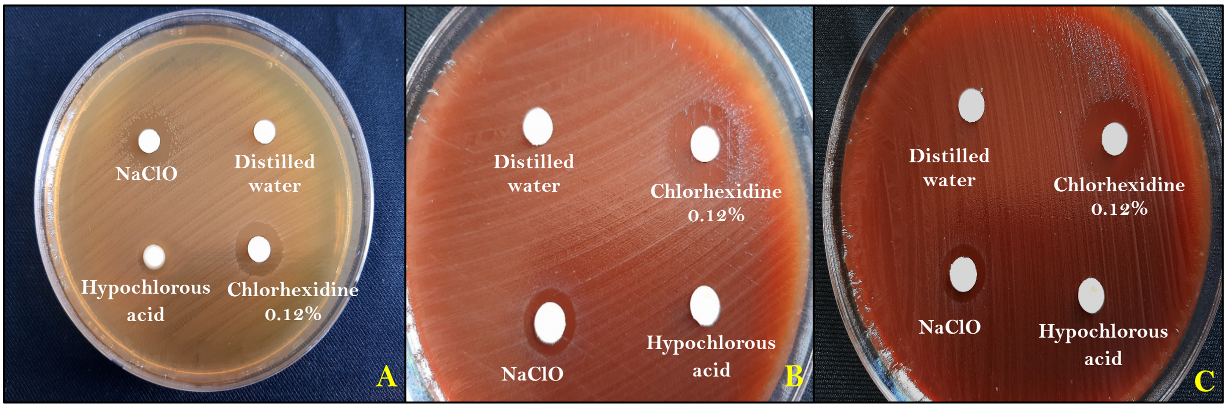

Figure 1: Agar plates with hypochlorous acid, 0.12% chlorhexidine, distilled water, and 4% sodium hypochlorite on S. gordonii (A), F. nucleatum (B), and P. gingivalis (C)

The range-based Kruskal-Wallis test showed differences in the zones of inhibition for the three oral bacteria *S. gordonii* ATCC 51656 (p=0.000), *F. nucleatum* ATCC 10953 (p=0.001), and *P. gingivalis* ATCC (p=0.000). The 0.12% CHX treatment showed greater control of the growth of *S. gordonii* ATCC 51656 (15.20 ± 0.66 mm.) and *P. gingivalis* ATCC (15.52 ± 0.43 mm.), but the effect was not significantly different from that of the NaClO treatment. In contrast, NaClO showed greater control of *F. nucleatum* ATCC 10953 (9.08 ± 4.53), but the effect was again not significantly different from that of 0.12% CHX.

Discussion

This study demonstrated that the 0.12% CHX and 4% NaClO groups showed bacterial reductions for the three bacteria evaluated. However, HOCl at 200 ppm was effective against only *S. gordonii*, a bacterium considered a primary colonizer in oral biofilms and responsible for biofilm adherence to surfaces [12,13]. Sarduy-Bermúdez and González Díaz [14] mentioned that these initial colonizers adhere to the film through specific molecules, called adhesins, that are present on the bacterial surface and interact with receptors in the dental film. HOCl appears to have a greater effect on bacteria that favor adherence than on late colonizers that constitute the biofilm.

Hypochlorous acid has uses in many industries, ranging from applications in agriculture and restaurants to medical care, including in the care and disinfection of chronic wounds [15,16]. In addition to its use as a liquid disinfectant, nebulization with hypochlorous vapor has been shown to have virucidal effects against several viruses and bacteria [17]. Numerous studies have confirmed that HOCl has efficacy in many clinical fields. In ophthalmology, HOCl in saline solution at a concentration of 100 ppm proved to be effective in decreasing the periocular bacterial load, reducing the staphylococcal load by 99% [18]; as a surface disinfectant, HOCl is effective at a concentration of 1000 ppm [19], and as a hand antiseptic, it has efficacy at a concentration between 100 and 200 ppm [20].

As a mouth rinse, HOCl has not been shown to have a systemic effect and appears to be safe for use in humans [21]. In other studies, HOCl has shown a broad antimicrobial spectrum for the inhibition of multiple microorganisms [22,23]. Castillo et al. [24] conducted an extensive investigation of the antimicrobial properties of a 0.050% and 0.0250% HOCl mouthwash and determined that HOCl was more effective than CHX (0.2%) against *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, and *Klebsiella oxytoca*.

In the present study, of the three bacteria evaluated, only *S. gordonii* showed an antibacterial effect. However, these results may not be very encouraging with respect to some antecedents [25,26]. This difference may be due to the different concentrations used, with different pH h in previous studies, the equipment used and even the inputs and volumes used in their preparation. Even so, it constitutes a starting point in a series of steps that must be rigorously followed to reach conclusions on its safety and efficacy.

Conclusion

Hypochlorous acid showed antimicrobial properties against only *S. gordonii* and was less effective than 4% sodium hypochlorite and 0.12% chlorhexidine, although no significant differences were found between the latter.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.		

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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