Viability and Effects on Bacterial Proteins by Oral Rinses with Hypochlorous Acid as Active Ingredient

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This study investigated the effect of hypochlorous acid (HOCl) rinses and chlorhexidine (CHX) on the bacterial viability of *S. mutans, A. israelii, P. gingivalis, A. actinomycetemcomitans, E. corrodens, C. rectus, K. oxytoca, K. pneumoniae* and *E. cloacae*. The percentage of live bacteria was tested by fluorescence method using Live/Dead kit® and BacLight (Molecular Probes®) and compared between groups by the Kruskal-Wallis and U Mann-Whitney tests with Bonferroni correction (p value<0.012). The effect of HOCl and CHX on total proteins of *P. gingivalis* and *S. mutans* was determined by SDS-PAGE. CHX showed a higher efficacy than HOCl against *S. mutans, A. israelii, E. corrodens* and *E. cloacae* (p<0.001) while HOCl was more effective than CHX against *P. gingivalis, A. actinomycetemcomitans, C. rectus* and *K. oxytoca* (p=0.001). CHX and HOCl had similar efficacy against *K. pneumoniae*. Proteins of *P. gingivalis* and *S. mutans* were affected similarly by HOCl and CHX. HOCl reduced the bacterial viability especially in periodontopathic bacteria, which may support its use in the control of subgingival biofilm in periodontal patients.

Introduction

Dental plaque is the most studied biofilm and it is the most common form of bacterial growth in the oral cavity (1). Many substances with antimicrobial effects have been developed and evaluated for the control of dental biofilm and gingivitis (2). Chlorhexidine (CHX) has been extensively studied in the inhibition of dental plaque, gingivitis reduction and is widely used in full-mouth disinfection protocols (3). However, CHX does not seem to be more effective than an essential-oil mouthwash to reduce gingivitis in long-term studies and its main action is the reduction of dental plaque (4,5).

There is a great interest in the development of new molecules with antimicrobial effect against oral microorganisms with a significant control of dental caries and periodontal disease. Hypochlorous acid (HOCl) is a non-antibiotic antimicrobial solution developed during the First World War by the dilution and acidification of sodium hypochlorite (Dakin solution) for the treatment of infected wounds (6). HOCl is part of a group of small molecules known as reactive oxygen species (ROS) synthesized by immune system cells during phagocytosis of antigens (7). The molecule has demonstrated a broad antimicrobial spectrum for the inhibition of multiple Gram positive and Gram negative microorganisms, without causing side effects such as irritation of the mucosa or extrinsic pigmentation on the tooth surface or restorations. HOCl also has an important anti-inflammatory and proliferative activity (8-10).

The purpose of this study was to evaluate the antimicrobial activity of hypochlorous acid rinses on oral bacteria (Gram positive and Gram negative) and study its effects on proteins of two of the major opportunistic and pathogenic oral microorganisms: *Porphyromonas gingivalis* and *Streptococcus mutans.*

Material and Methods

HOCl Preparation

A special formulation of HOCl for oral health was developed according to formulations and technology patented by Aquilabs S.A. (US patent: US2009/0258083A1) (11). The solutions were obtained with a concentration of available chlorine as HOCl of 250 ppm (0.025%) and 500 ppm (0.05%) according to NTC 1847 with an ORP (oxide reduction potential) of 950-1100 MV and a conductivity of 25.3 ds/m, at a density of 1.01 g/mL at pH 5.8 ± 0.2. HOCl solution was subjected to an accelerated stability test to ensure a stable solution for more than 12 months before losing 10% of its active ingredient.

Antimicrobial Mechanisms

The viability for *Streptococcus mutans* ATCC 25175, *Actinomyces israelii* ATCC 12012, *Porphyromonas gingivalis* ATCC 33277, *Aggregatibacter actinomycetemcomitans* ATCC 29523, *Eikenella corrodens* ATCC 23834, *Campylobacter rectus* ATCC 33238, *Klebsiella oxytoca* ATCC 43086, *Klebsiella pneumoniae* ATCC 700603, *Enterobacter cloacae* ATCC 13047 was tested by fluorescence method using Live/

Dead kit® and BacLight (Molecular Probes®).

Bacterial Culture and Inoculum Standardization

ATTC strains of *P. gingivalis*, *A. israelii*, *E. corrodens* and *C. rectus* were grown in supplemented brucella agar (0.3% Bacto agar, 0.2% yeast extract, 5% defibrinated sheep blood, 0.2% hemolyzed blood, 0.0005%, hemin, 0.00005% and menadione) and incubated at 37 °C for 4 days in anaerobic conditions (Anaerogen, Oxoid, Hampshire, UK). *A. actinomycetemcomitans* was seeded in agar Dentaid 1 and incubated at 37 °C for 72 h with 5-10% CO_2. *S. mutans* was grown in blood agar (Blood Agar Base with 5% sheep blood) and incubated at 37 °C for 24 h with 5-10% CO_2. *K. oxytoca*, *K. pneumoniae* and *E. cloacae* were plated on BHI agar (Brain Heart Infusion) and incubated for 24-48 h at 37 °C in aerobic atmosphere. Subsequently, inocula were performed in BHI broth (Brain Heart Infusion) and were quantified by spectrophotometry for specific optical densities (OD) of 1 x 10^8 cells/mL or higher according to Time Kill Assay protocol (12). After obtaining the expected OD base 10, serial dilutions were plated on appropriate agar for each type of microorganism and incubated under the conditions referred above, to confirm count colony forming units (CFU). Assays were made in triplicate and the results are shown as mean and standard deviation (Table 1).

Bacterial Viability Test

To evaluate the number of live and dead bacteria after exposure to different concentrations of HOCl and CHX 0.2%, the inocula of all previously described bacteria were adjusted to a 1 x 10^8 bacteria/mL concentration and cultured in BHI broth. 230 µL of each bacteria in culture were exposed by for 30 s to 700 µL of HOCl at 250 ppm and 500 ppm concentrations at pH=5.8. Reactions were neutralized with 70 µL of sodium thiosulfate 0.10 N and the mixture (HOCl + bacteria + neutralizer), and centrifuged at 14,000 rpm during 5 min. The pellet was re-suspended in 50 µL of the supernatant and the excess was discarded. 0.15 µL of the viability mixture (Live/Dead® BacLight Bacterial Viability) was added to the 50 µL pellet. The mixture contains two dyes: SYTO 9 3.34 mM and propidium iodide 20 mM. Samples were incubated in low-light conditions for 15 min. The dyes were used to differentiate the bacteria with intact membrane (green fluorescence) from the bacteria with abnormal membrane (red fluorescence). Positive control with CHX 0.2% and negative control without treatment were used in the same experimental conditions. The laminas were preincubated with 2 µL of formaldehyde at 2%, in order to immobilize the bacteria. Images were observed in a fluorescence microscope (Axio-Imager A2; Zeiss, Jena, Germany) in greater magnification and digitized using the AxioVision LE 4.8 software (Zeiss Microscopy). Quantification was determined by the number of pixels for live and dead bacteria using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and data were expressed as percent of viability.

Evaluation of the Protein Integrity

P. gingivalis ATCC 33277 and *S. mutans* ATCC 25175 were cultured as described above. A suspension of each bacterium was made in BHI broth and standardized to a concentration of 1 x10^8 bacteria/mL and adjusted spectrophotometrically as above described. Bacterial suspensions were exposed to HOCl at 250 ppm and 500 ppm at pH=5.8. CHX 0.2% was used as positive control and untreated inoculum as negative control. The cell pellet was washed in PBS (Phosphate Buffer Saline) and re-suspended in 2 mL of lysis buffer (50 mM Tris pH=7.5, 50 mM NaCl, 5% glycerol). For *P. gingivalis*, bacterial inhibitor and proteinase K (10 mg/mL) was added and incubated for 15 min at 65 °C. For *S. mutans*, buffer plus bacterial protease inhibitor and 20 mg/mL of lysozyme was added and incubated at 37 °C overnight. The samples ere sonicated on ice in a Sonics Vibra-Cell VCX 130 ultrasonic processor (Sonics & Materials, Inc. Newtown, CT, USA) with 40% amplitude, totalizing 7 cycles (30 s on, 15 s off). The extracts were stored at -20 °C.

The protein concentration was determined using the bicinchoninic acid reagent (BCA) to quantify the outer membrane protein by a colorimetric method (13) and determining the concentration of the protein according to

Table 1. Standardized bacterial inoculum

Bacteria	Wavelength	OD ± 0.02	Bacteria/mL (CFU) mean ± SD x 10^8
Streptococcus mutans ATCC 25175	580 nm	0.570	1.5±0.7
Actinomyces israelii ATCC 12012	600 nm	1.000	1±0
Klebsiella oxytoca ATCC 43086	580 nm	0.700	1.66±0.5
Klebsiella pneumoniae ATCC 700603	580 nm	1.000	1.66±1.5
Enterobacter cloacae ATCC 13047	580 nm	0.980	1±0.5
A. actinomycetemcomitans ATCC 29523	480 nm	1.700	2.33±1.5
Porphyromonas gingivalis ATCC 33277	620 nm	0.900	1.66±1.1
Eikenella corrodens ATCC 23834	620 nm	1.500	1.5±0.7
Campylobacter rectus ATCC 33238	620 nm	1.000	1.33±0.5

the manufacturer's instructions (Thermo Specific).

The integrity of the proteins was evaluated using electrophoresis in polyacrylamide gel in 10% SDS (sodium dodecyl sulfate) and a standard pattern of known molecular weight was used to determine the molecular weights of the different bacterial proteins. Separating gels (12%

acrylamide/bisacrylamide) were used and the samples diluted in Laemmli buffer in each well with the same protein concentration (20 µg/µL) to evaluate the differences in the electrophoretic profile of the bacteria treated with the different HOCl concentrations. A run buffer with 3% Tris base, 14% glycine, 1% SDS, pH=8.3 was used and the gels were run for 80 min at 100 V. Past the runtime each gel was stained using Silver Stain Kit Pierce® (Thermo Fisher Scientific, Inc., Waltham, MA, USA.), following the manufacturer's recommendations.

Oral bacterial viability with hypochlorous acid

Statistical Analysis

The average of live bacteria for each experiment was calculated as percentage. The viability reduction was calculated as the difference of living bacteria between the untreated controls per experiment. Descriptive analysis of the means and standard deviations were made. Viability percentages between different rinses were compared through the Kruskal Wallis and U Mann-Whitney test with Bonferroni correction. A p-value of <0.012 was established for differences between groups.

Results

Inoculum Standardization

Table 1 shows the average and standard deviation of CFU counts for reference strains used in the standardization phase of the bacterial inoculum, required to perform the tests at a concentration of 1×10^8 bacteria/mL or higher according to the recommendations of the ASTM E2315-03 for the Time Kill Method for antimicrobial agents (12).

Bacterial Viability Assessment

Viability percentages for Gram positive observed in Fig.1A. The viability of *S. mutans* and *A. israelii* was significantly affected by CHX to 0.2% and HOCl at 500 ppm when compared with the untreated control and with HOCl at 250 ppm (p=0.001). However, 0.2% CHX showed a higher efficacy against these microorganisms when compared with all groups (p<0.001). HOCl 250 ppm showed greater effect than the control for *A. israelii* (p=0.0032), but did not show differences against *S. mutans* (p= 0.64) (Fig. 1). 500 ppm HOCl showed to be the most effective substance reducing the viability of Gram
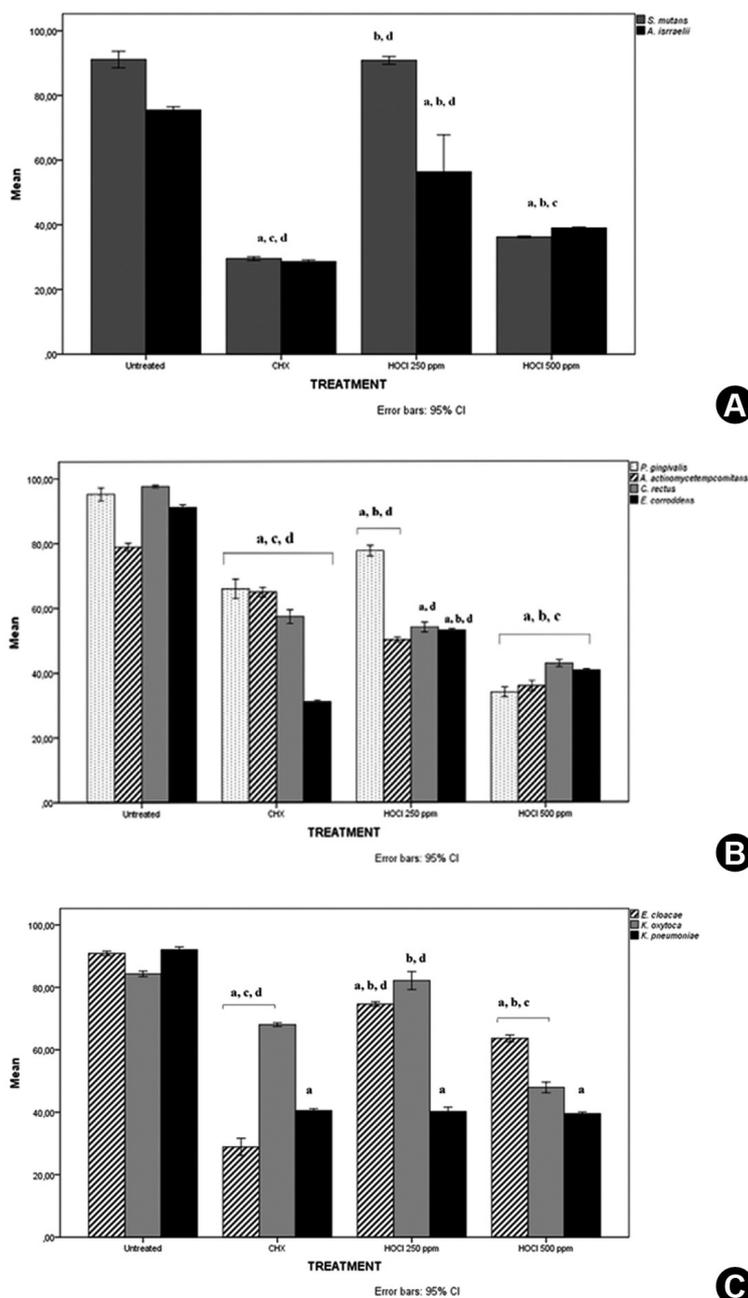
Figure 1. Percentage of bacterial viability after 30 seconds treatment with CHX, HOCl de 250 ppm and 500 ppm. A: Gram positive bacteria: *S. mutans* ATCC 25175 and *A. israelii* ATCC 12012. B. Gram negative bacteria associated to periodontal disease: *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 29523, *C. rectus* ATCC 33238 and *E. corrodens* ATCC 23834. C. Enteric rods: *E. cloacae* ATCC 13047, *K. oxytoca* ATCC 43086 and *K. pneumoniae* ATCC 700603. p-values come from t-test or Kruskal Wallis y U Mann-Whitney (p≤0,012). a: p-value for differences with untreated; b: p-value for differences with chlorhexidine; c: p-value for differences with HOCl 250 ppm; d: p-value for differences with HOCl 500 ppm.

D.M. Castillo et al.

negative organisms associated with periodontal disease. *P. gingivalis, A. actinomycetemcomitans* and *C. rectus* showed significant differences for HOCl at 500 ppm in all evaluated groups including CHX 0.2% (p=0.001). CHX 0.2% showed better effectiveness in *E. corrodens* compared to all groups (p<0.001). However, HOCl to 250 ppm and 500 ppm were more effective than the control group for this microorganism (p<0.001) Figure 1B. Viability of *P. gingivalis* after treatments is observed in Figure 2.

The enteric rods commonly found as contaminants in samples of saliva and subgingival plaque were affected by the solutions in study. *K. pneumoniae* showed a similar reduction to 0.2% CHX and the two concentrations of HOCl (p>0.05). *K. oxytoca* showed reduced viability for HOCl 500 ppm for all evaluated groups (p<0.001). CHX 0.2% showed a better efficacy against *K. oxytoca* than HOCl at 250 ppm and the control group (p<0.001). *E. cloacae* showed a significant decrease in bacterial viability for 0.2% CHX compared to all evaluated groups (Fig. 1C).

Evaluation of the Protein Integrity

After the treatment with HOCl at 500 ppm, 250 ppm and CHX there was an important change in the banding pattern in bacterial proteins for the reference strains of *P.*

gingivalis and *S. mutans.* A disappearance of a large part of the bands is observable compared to the untreated control.

For *P. gingivalis* some of the low molecular weight proteins are conserved, on the other side the high molecular weight proteins lose their sharpness as if the proteins concentrations decreased after the treatment with HOCl. After treatment with CHX thin proteic profile is observed. However the protein concentration seems to be affected, suggesting some kind of protein alterations, but less than with HOCl. In *S mutans* is more evident the disappearance of the bands with high molecular weight and a band of approximately 28 kD is conserved for all treatments. A band of 70 kD for the HOCl at 250 ppm is conserved but in minor concentration. All experiments were made by triplicate and presented the same behavior (Fig. 3).

Discussion

There is an agreement on the effect of various antiplaque substances to reduce gingivitis in long term studies (4). However, the effective reduction of plaque and gingivitis in the short term is still under study. Chlorhexidine remains as the gold standard as antiplaque agent in short and long term effect (5). Despite their wide use, some adverse effects have discouraged its use, as tooth discoloration as it is easily
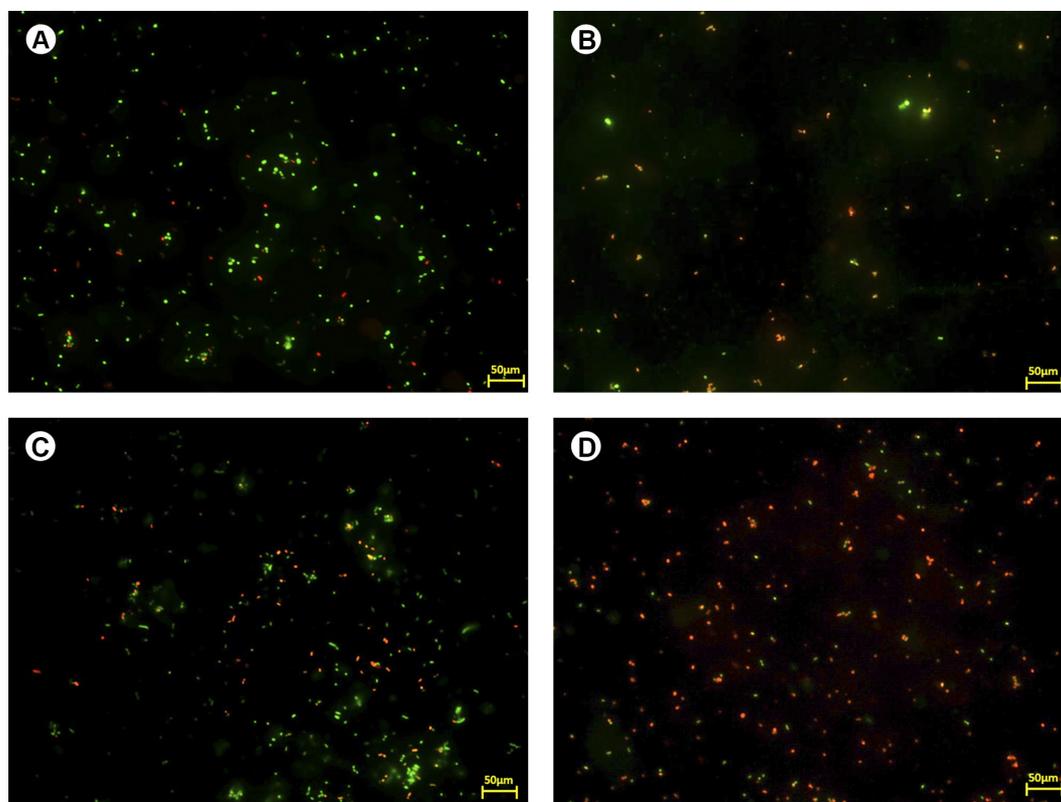

Figure 2. Bacterial viability of *P. gingivalis* ATCC 33277. Green fluorescent bacteria are alive and red fluorescing bacteria are dead. A: untreated; B: 0.2% chlorhexidine; C: HOCl 250 ppm, pH 5.8 and D: HOCl 500 ppm, pH 5.8.

mixed with the dietary chromogens, weak microbicidal activity at low concentrations and at high concentrations may produce dermatitis and desquamation of oral mucosa as well as delay in healing (14,15).

Hypochlorous acid rinses are proposed for plaque control and as a wound healing agent for its use in oral health, due to its low toxicity, high antimicrobial efficacy, anti-inflammatory effect, induction to cell proliferation and its background as a topical solution in the antisepsis of wounds in clinical medicine (16). HOCl has also the ability to oxidize the amino acid taurine and induce the formation of chlorine-taurine (TauCl) which has broad spectrum antimicrobial activity. The TauCl has a significant protective effect on tissues because it can inhibit the production of inflammatory mediators and thereby contribute to the processes of tissue protection (10).

In this study, HOCl showed a significant effect on Gram positive bacteria but did not exceed the effect of chlorhexidine. Chlorhexidine has shown better effect on Gram positive microorganisms but less on Gram negative microorganisms (17,18). The antimicrobial action of HOCl appears to be greater in Gram negative than in Gram positive possibly because Gram negative bacteria has sulfur and hem groups (rich in iron) in its membrane which causes an irreversible reaction HOCl/membrane proteins, producing structural damage, and altering cell permeability, affecting bacterial viability in Gram negative bacteria (19,20). The HOCl oxidizes and/or chlorinates endotoxins and exotoxins such as lipopolysaccharides and gingipains as Rgp and Kgp neutralizing their action. In Gram positive bacteria HOCl oxidizes glycine residues present in the peptidoglycan, on the other hand chlorination reactions in this group of microorganisms differs in the action on the target (21).

Different authors have controversial uses of microbiological culture methods to evaluate the efficacy of antimicrobials. The bacterial viability assessment with specific methods such as fluorochromes or epifluorescence has been proposed for evaluation of antimicrobials (22). In the present study a method of epifluorescence was used for the evaluation of the bacterial viability similar to the reported by other studies that have evaluated the substantivity of antiplaque substances (23,24).

Proteins of *P. gingivalis* and *S. mutans* were affected after 30 s of treatment with the test solutions. For *P. gingivalis*, after HOCl and CHX treatments it is observed a similar reduction of the protein concentrations in many bands when is compared with the untreated control. In *S. mutans* there is an elimination of almost all the proteins for the different treatments when compared with the untreated control. Cheung et al., in 2011 have shown changes in the proteic profile in *Bacillus subtilis* and *E. coli* after treatment with CHX, suggesting that the mechanism of action of CHX is related with an alteration of lipidic stability of the cell membrane (25). It is not clear which is the action of HOCl on bacteria, however oxidation and chlorination of amino groups in some functional and structural proteins in Gram positive and Gram negative bacteria is suggested (7). In further it is important to identify the proteins affected by HOCl action and thus explain how this antimicrobial molecule affects the bacterial viability and why in some Gram negative bacteria as *E. cloacae* does not have an important reduction in the viability.

The formulation of HOCl has been stabilized in Colombia and patented as an substance with antimicrobial effects

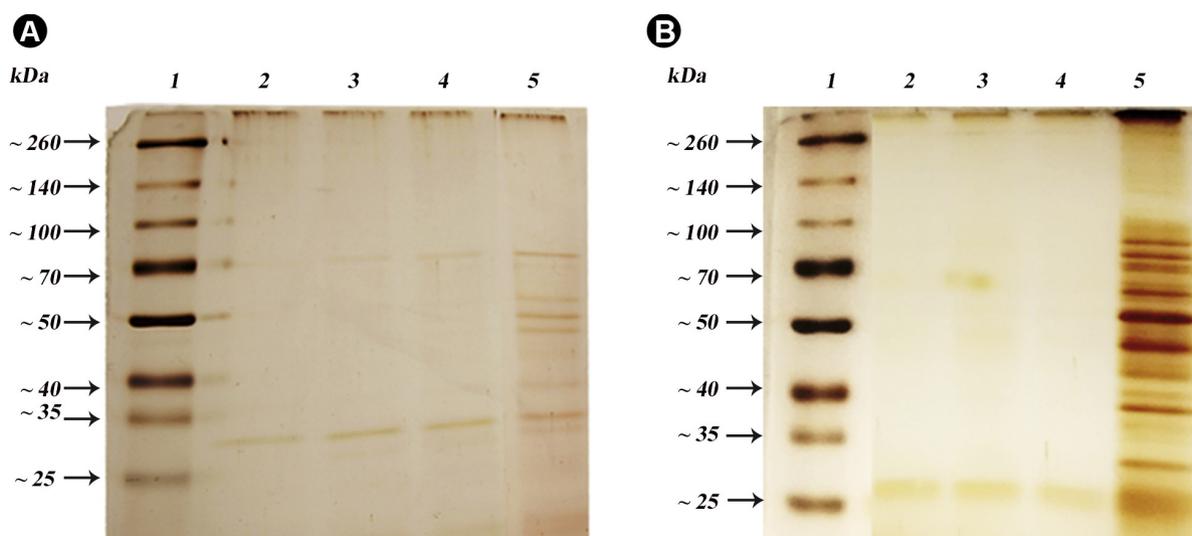*Oral bacterial viability with hypochlorous acid*

Figure 3. Total protein electrophoretic profile of *P. gingivalis* ATCC 33277 (A) and *S. mutans* ATCC 25175 (B). Lanes: 1 molecular weight marker; 2. Treatment with HOCl 250ppm; 3. Treatment with HOCl 500ppm; 4. Treatment with CHX; 5. Without treatment.

D.M. Castillo et al.

for medical applications such as the treatment of chronic and non-healing wounds (11). Findings of this study could support future research of HOCl as antimicrobial and antiplaque agent in dentistry.

HOCl showed better effects on bacterial viability than CHX in Gram negative microorganism specially in *P. gingivalis*, *A. actinomycetemcomitans* and *C. rectus*. HOCl could have a significant effect on periodontophatic bacteria that could colonize and aggregates as dental biofilm.

Resumo

Este estudo investigou o efeito de enxaguantes à base de ácido hipocloroso (HOCl) e clorexidina (CHX) sobre a viabilidade bacteriana de *S. mutans, A. israelii, P. gingivalis, A. actinomycetemcomitans, E. corrodens, C. rectus, K. oxytoca, K. pneumoniae* e *E. cloacae*. O percentual de bactérias sobreviventes foi testado pelo método de fluorescência utilizando Live/Dead kit® e BacLight (Molecular Probes®), fazendo comparação entre os grupos com os testes de Kruskal-Wallis e U Mann-Whitney e correção de Bonferroni (p<0,012). O efeito de HOCl e CHX sobre *P. gingivalis* e *S. mutans* foi determinado por SDS-PAGE. O CHX mostrou eficácia superior ao HOCl contra *S. mutans, A. israelii, E. corrodens* e *E. cloacae* (p<0,001), ao passo que *P. gingivalis, A. actinomycetemcomitans, C. rectus* e *K. oxytoca* foram melhores que o CHX para o HOCl (p=0,001). O *K. pneumoniae* teve efeito similar para o CHX e para o HOCl. As proteínas de *P. gingivalis* e *S. mutans* foram afetadas de modo semelhante por CHX e HOCl. O HOCl reduziu a viabilidade bacterial, especialmente nas bactérias periodontopáticas, o que pode recomendar o uso no controle do biofilme subgingival em pacientes periodontais.

Acknowledgements

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