The Effect of 0.12% Chlorhexidine Gluconate Rinsing on Previously Plaque-Free and Plaque-Covered Surfaces: A Randomized, Controlled Clinical Trial

Fabrício B. Zanatta,*† Raquel P. Antoniazzi,* and Cassiano K. Rösing*

Background: Previous in vitro studies showed little bactericidal effect on structured oral biofilm after exposure to chlorhexidine (CHX). In vivo evidence of a CHX effect against structured biofilm is scarce. The purpose of this study was to compare the efficacy of 0.12% CHX gluconate on previously plaque-free and plaque-covered surfaces.

Methods: This study had a single-masked, randomized split-mouth, 21-day experimental gingivitis design including 20 individuals who refrained from all mechanical plaque control methods for 25 days. On day 4 of plaque accumulation, the individuals had two randomized quadrants cleaned; the other two quadrants served as the plaque-covered surfaces. Also, on day 4, the individuals started rinsing with 0.12% CHX gluconate for 21 days. The Quigley and Hein plaque index (PI), gingival index (GI), and gingival crevicular fluid (GCF) volume were assessed at baseline and days 21 and 25. The PI also was assessed at days 4, 11, and 18.

Results: Intergroup comparisons showed statistically higher PI throughout the study on the plaque-covered surfaces compared to the plaque-free surfaces. When the inflammatory response over time was analyzed, a statistically greater increase in GI (from 0.21 ± 0.02 to 0.93 ± 0.03 versus from 0.18 ± 0.01 to 0.52 ± 0.03 on plaque-covered and plaque-free surfaces, respectively) and GCF volumes (from 48.09 to 94.28 μl versus from 46.94 to 64.99 μl on plaque-covered and plaque-free surfaces, respectively) occurred on plaque-covered surfaces after 21 days of plaque accumulation.

Conclusions: A 0.12% CHX gluconate mouthrinse had little antiplaque and antigingivitis effect on previously plaque-covered surfaces. These results confirm the diminished effect of CHX on structured biofilm and reinforce the necessity of biofilm disruption before the initiation of CHX mouthrinse. *J Periodontol 2007;78:2127-2134.*

KEY WORDS

Biofilm; chlorhexidine; dental plaque; gingival crevicular fluid; gingivitis.

Dental plaque is a specific type of biofilm formed by the interaction of salivary coating and microbial deposition embedded in an extracellular polysaccharide matrix. These microorganisms are adherent to each other and/or to surfaces as a result of the dynamic balance between microbial attachment processes and mechanical forces of detachment in the oral cavity.[1-6] Epidemiologic surveys[7-9] showed that a definite relationship exists between dental biofilm and periodontal disease.

The accumulation and maturation of bacterial biofilm at the gingival margin is widely recognized as the primary etiologic factor in the development of chronic gingivitis; meticulous oral hygiene can restore gingival health.[10-14] Based upon this association, current treatment for gingivitis is directed at disruption of biofilm, which usually includes professional and homecare mechanical methods.[15,16] However, it is not easy to achieve an adequate level of plaque control; efficient plaque-control techniques are time-consuming and require motivation and skill to be performed well.[17]

Several antimicrobial agents have been incorporated in mouthrinses to improve the outcome of mechanical oral hygiene procedures or even to replace mechanical plaque control. Chlorhexidine (CHX) has

* Postgraduate Program in Dentistry, Lutheran University of Brazil, Canoas, RS, Brazil.
† School of Dentistry, Franciscan University Center, Santa Maria, RS, Brazil.

doi: 10.1902/jop.2007.070090

been established as the most effective chemical plaque-control compound.[18-22] It is a broad-spectrum antiseptic with pronounced antimicrobial effects on Gram-positive and -negative bacteria as well as on fungi and some viruses.[23] CHX is a positively charged bisbiguanide that can adsorb to different negatively charged sites, including mucous membranes, salivary pellicle on teeth, and titanium surfaces, as well as several components of the biofilm on the tooth surfaces, e.g., bacteria, extracellular polysaccharides, and glycoproteins.[24-27]

In vitro studies showed that, in low concentrations, CHX causes damage to the cell membrane, and low molecular weight molecules escape from the microorganisms. At higher concentrations, CHX causes precipitation and coagulation of the proteins in the cytoplasm of the exposed microbes.[28-30] These properties interfere with biofilm formation and prevent the growth processes. Unfortunately, most laboratory studies[4-6] of the potential effectiveness of such agents applied determination of the minimum inhibitory concentration, which is not adequate if one understands the nature of biofilms as a complex community with a profound evolutionary process.

Recent evidence[4,31,32] suggested that bacterial phenotypes may be modified when the organisms change from a planktonic to a sessile state (as part of a biofilm). These changes can result in altered susceptibilities to antimicrobial agents.[33] Pratten et al.[34] demonstrated that 0.2% CHX gluconate had little effect on the viability of established biofilms in vitro after pulsing twice daily for 4 days. Other in vitro studies[35,36] demonstrated a small effect of CHX on developed biofilm viability.

Several clinical trials[21,37-46] demonstrated that CHX is effective in reducing the formation of dental plaque and preventing gingivitis. However, these studies involved previously disrupted biofilms on all dental surfaces. Considering the difficulty of CHX to kill microorganisms in an undisturbed biofilm, it is necessary to investigate whether CHX is able to reduce the established biofilm and prevent the establishment of gingivitis around tooth surfaces harboring dental plaque.

The aim of the present study was to compare the effects of 0.12% CHX gluconate in plaque and gingival inflammation in sites with and without supragingival biofilm that was established in an experimental gingivitis model.

MATERIALS AND METHODS

The present study was a randomized split-mouth clinical trial using the experimental gingivitis model proposed by Löe et al.[10] The study protocol was submitted to and approved by the Ethical Committee of the Lutheran University of Brazil in April 2006, and the study was conducted between May and July 2006.

Test Panel

The test panel consisted of 20 subjects recruited from dental students at the Lutheran University of Brazil. To assess the power of the present study, we used the plaque index (PI) as the primary outcome. Taking into consideration the paired design, we considered a mean difference of 0.60 (with a standard deviation of 0.25) as clinically relevant. Accepting an α error of 0.05, the power of this study was 0.88. The mean age of the volunteers was 28.15 ± 3.15 years. At recruitment, subjects were asked about their medical and dental histories. Written and oral explanations detailing the study purpose and design were given to each subject. Subjects who preliminarily met inclusion/exclusion criteria were selected for a dental screening appointment. If the subject met all of the inclusion/exclusion criteria, an informed consent was provided.

Inclusion criteria included: age between 18 and 35 years; male gender; no relevant medical conditions that could interfere with the periodontal health; probing depth <3 mm and clinical attachment loss <2 mm at all sites; and willingness to comply.

Exclusion criteria included: antibiotic and/or anti-inflammatory therapy within 3 months prior to baseline examination; oral mucosal lesions; smoking; need for antibiotic premedication; history of hypersensitivity to CHX; and any factor that could retain plaque, e.g., carious lesions, inadequate restorations, dental implants, orthodontic appliances, and fixed or removable prostheses.

Exclusion criteria during the study included individuals who wanted to be released from the study; any acute process, such as allergic reaction to the product or gingival abscess; necessity for any antibiotic or anti-inflammatory; use of any rinsing product other than the CHX rinses; and individuals who did any mechanical biofilm control.

Clinical Parameters

The following clinical parameters were obtained in the order listed below from all teeth, except third molars. Quigley and Hein[47] PI, as modified by Turesky et al.,[48] was scored as 0 = no plaque; 1 = separate flecks of plaque at the cervical margin of the tooth; 2 = a thin continuous band of plaque (≤1 mm) at the cervical margin of the tooth; 3 = a band of plaque wider than 1 mm but covering less than one-third of the crown of the tooth; 4 = plaque covering at least one-third but less than two-thirds of the crown of the tooth; and 5 = plaque covering two-thirds or more of the crown of the tooth.

Before scoring, an air/water spray was used to remove any food. All quadrants were stained by topically applied 75% sodium fluorescein solution with a swab, taking care not to disrupt the existing

supragingival biofilm. The teeth were dried using a chair-side air syringe and isolated carefully with cotton rolls. Plaque was disclosed and measured using a blue light.[49]

Gingival crevicular fluid volume (GCF) was collected according to the recommendations of Deinzer et al.[50] and measured with an electronic gingival fluid measuring device.‡ This was performed according to the manufacturer's instructions at two sites per quadrant (upper lateral incisor = labial site; upper first premolar = mesio-buccal site). The area was isolated carefully with cotton rolls to avoid salivary contamination, and a paper strip was introduced into the crevice until mild resistance was achieved. Care was taken to avoid any mechanical injury to marginal tissues. The strip was left in place for 3 minutes and was transferred immediately to the calibrated electronic gingival fluid measuring device. The examinations were performed in a climate-controlled room (~20°C) to minimize the possible impact of the room temperature and humidity on the sample volume.

The gingival index (GI), according to a modification proposed by Löe,[51] was measured at six sites per tooth (disto-buccal/labial, bucco-buccal/labial, mesio-buccal/labial, disto-lingual/palatal, linguo-lingual/palatal, and mesio-lingual/palatal). It was scored as 0 = absence of visual alterations of marginal gingiva; 1 = visual alterations of marginal gingiva; 2 = bleeding upon gentle probing; and 3 = tendency to spontaneous bleeding.

Calibration of the Electronic Gingival Fluid Measuring Device

Calibration of the electronic gingival fluid measuring device was performed with a standardized syringe, and 0.1 to 0.8 μl distilled water in increments of 0.1 μl was applied to paper strips to determine the precision of the calibration results. The test fluid was drawn into the syringe repeatedly (three times) and then dispensed into the vial to ensure that the inner walls of the syringe were coated with fluid, thereby minimizing pipetting error. A quadratic regression curve was computed as the standard curve for the determination of unknown GCF volumes. If the mean results of these repeated analyses differed by >5 units, the electronic gingival fluid measuring device was recalibrated.

Assessment of Intraexaminer Reproducibility

Before starting the trial, multiple sessions of training for the assessment of clinical parameters were performed with a calibrated professional. Disagreements were resolved by discussion. After the examiner was trained according to the calibrated professional's criteria, the intraexaminer reproducibility was assessed. For calibration, four dental students were recruited who were not included in the study population. Students were evaluated during two sessions: one for PI measurement and one for GI measurement. The examiner scored all teeth during each session. A duplicate examination was performed 1 hour later. The PI and GI were compared to κ statistics.

The κ coefficient was 0.88 and 0.93 for PI and GI, respectively. After half of the sample was included in the study, new intraexaminer reproducibility was assessed using two subjects (10% of the total sample). The reproducibility analysis was conducted in the same way as the first one; the κ coefficient was 0.78 and 0.84 for PI and GI, respectively.

Study Design

The outline of the experimental procedures is summarized in Figure 1.

To achieve optimal gingival health and to standardize gingival baseline conditions, all subjects participated in a 14-day pretrial period. This visit consisted of professional scaling and polishing with a rubber cup and prophylactic paste§ and dental floss. If their hygiene techniques were judged insufficient, individual instructions were given on how to improve performance.

On day 0 (baseline), PI, GCF, and GI were recorded in all subjects. After the recordings, a prophylaxis was performed and detailed explanations to stop all plaque control were provided.

On day 4, each subject had one upper and one lower quadrant assigned randomly as "tests" (established supragingival biofilm was not removed), and the other quadrants were considered the "controls" (established supragingival biofilm was removed professionally with rubber cup, prophylactic paste, and dental floss). After the prophylaxis on the control quadrants, the PI was recorded in all teeth to ensure that the control quadrants were free of supragingival biofilm. On this day, all subjects continued the oral hygiene withdrawal for 21 days and started to rinse twice daily with 15 ml mouthrinse containing 0.12% CHX gluconate.‖

On days 11 and 18, the PI was scored for all teeth (six sites per tooth). On day 25, PI, GCF, and GI were recorded for all teeth. After the recordings, a prophylaxis was performed, and all subjects were free to return to their normal habits of mechanical plaque control. Gingivitis was treated if necessary.

Examiner Masking

On days 11, 18, and 25, the examiner was kept unaware of the randomization sequence and was masked as to which quadrant was test or control. However, because of the nature of the study, it was not possible to assume that the examiner had not become aware of quadrant allocation; plaque accumulation was

‡ Periotron 8000, Harco Electronics, Winnipeg, MB.
§ K.G. Sorensen, São Paulo, SP, Brazil.
‖ Noplak, Daudt, São Paulo, SP, Brazil.

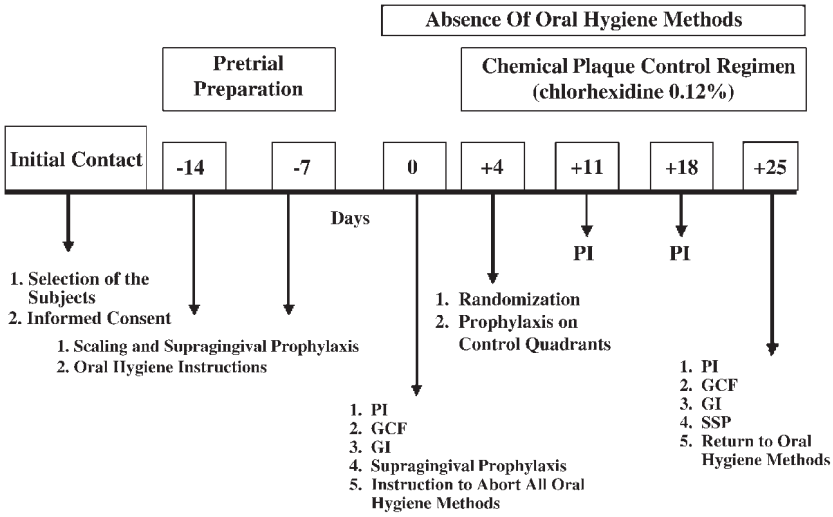

Figure 1.
Experimental design.

Table 1.

PI During the Experimental Period for Initially Plaque-Covered and Plaque-Free Surfaces

	Plaque-Free Surfaces (control)		Plaque-Covered Surfaces (test)	
PI	Mean	SE	Mean	SE
Day 0	0.13A*a†	0.02	0.16Aa	0.03
Day 11	0.20Ab	0.02	1.67Bb	0.07
Day 18	0.30Ac	0.02	1.15Bc	0.07
Day 25	0.43Ad	0.03	1.03Bd	0.05

* Uppercase letters refer to the comparison between initially plaque-free and plaque-covered surfaces in each experimental period. Different letters demonstrate statistically significant differences (Wald test; $P<0.05$).
† Lowercase letters refer to within-group comparison over time. Different letters demonstrate statistically significant differences (Wald test; $P<0.05$).

clinically different between test and control quadrants, especially on days 7 and 14. It is unknown whether this may have biased the examiner.

Checking of Compliance

The necessary volume of CHX for the entire experimental period was 630 ml (15 ml two times a day; 12/12 hours during 21 days). To check compliance, >630 ml CHX solution was given in 1-liter flasks, with different volumes of the antiseptic in each flask. At the end of the experimental period, all subjects returned the remaining CHX solution to check individual adherence to the CHX rinsing.

Statistical Analysis

Data analysis was performed using commands that take into account clustering of observations within subjects.¶ A robust variance estimator was used to adjust for the clustering of teeth (site analysis) into individuals. Wald tests were used for comparisons, and the *P* value was adjusted for multiple comparisons. The individual was the unit of analysis, and the level of significance was set at 5%.

RESULTS

All 20 subjects who fulfilled the inclusion/exclusion criteria completed the study. Checking of compliance revealed that 100% of the individuals used the expected amount of CHX.

Table 1 shows the mean PI in both groups throughout the study. At baseline, no statistically significant differences were detected between the groups. An increase in mean PI was observed for initially plaque-free and plaque-covered surfaces; however, the magnitude of the increase was higher in the plaque-covered surfaces. Moreover, intergroup comparisons revealed that the initially plaque-covered group exhibited higher mean values of PI on days 11, 18, and 25.

To further examine the role of CHX in preventing gingival inflammation on initially plaque-free and plaque-covered surfaces, the mean GI scores were compared. In initially plaque-free quadrants, mean GI values increased significantly ($P<0.05$) from 0.18 ± 0.01 to 0.52 ± 0.03 on days 0 and 25, respectively. In initially plaque-covered quadrants, these values increased from 0.21 ± 0.02 to 0.93 ± 0.03, respectively, also a statistically significant difference. The comparison of mean GI at baseline did not display statistically significant differences between groups; however, at day 25, initially plaque-covered surfaces showed higher degrees of inflammation (Fig. 2).

Changes in marginal bleeding, as assessed by GI ≥2, are shown in Figure 3. Both groups had significantly increased marginal bleeding. However, after 3 weeks of CHX mouthrinses, percentages of GI = 2 were significantly higher in initially plaque-covered surfaces compared to plaque-free surfaces.

The analysis of GCF volume is demonstrated in Figure 4. At baseline, no significant differences were observed between the groups ($P>0.05$). Both groups had a statistically significant increase in mean CGF from day 0 to day 25 (from 46.94 to 64.99 μl in

¶ Stata 9.2 for Windows, Stata, College Station, TX.

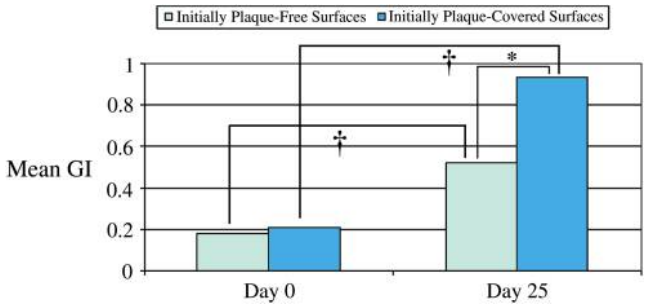

Figure 2.
*Mean GI scores in teeth with and without plaque on days 0 and 25. *Statistically significant differences between initially plaque-free and plaque-covered surfaces (Wald test; P = 0.05). †Statistically significant differences observed over time in plaque-free (Wald test; P = 0.05) and plaque-covered (Wald test; P = 0.05) surfaces.*

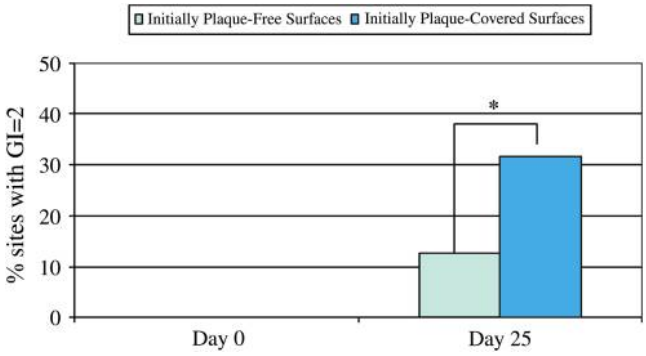

Figure 3.
*Percentage of sites with marginal bleeding (GI = 2) in teeth with and without plaque on days 0 and 25. *Statistically significant differences between initially plaque-free and plaque-covered surfaces (Wald test; P = 0.05).*

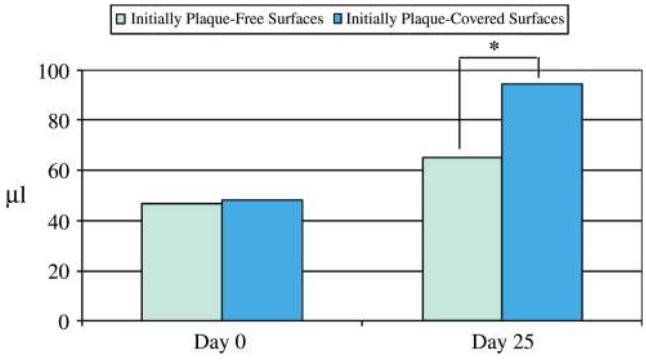

Figure 4.
*Mean GCF volume (µl) in teeth with and without plaque on days 0 and 25. *Statistically significant differences between initially plaque-free and plaque-covered surfaces (Wald test; P = 0.05).*

plaque-free surfaces and from 48.09 to 94.28 µl in plaque-covered surfaces). At day 25, a statistically significant difference was observed between the groups.

DISCUSSION

The purpose of the present investigation was to answer an often-asked clinical question with scarce evidence in humans: What is the efficacy of CHX on teeth with established biofilm? To test the hypothesis of no difference in the effect of CHX usage on initially plaque-covered or plaque-free surfaces, a randomized split-mouth clinical trial was designed.

The methods used in our study were chosen in an attempt to reduce variability and increase validity. Using dental students in the investigation was aimed at having the best possible gingival health. An experimental gingivitis model was used to evaluate the impact of professional prophylaxis on gingival status over a 21-day period. The split-mouth model used in our study could evaluate, in the same person, the gingival response to CHX in previously plaque-free and plaque-covered surfaces, avoiding a comparison between different inflammatory response patterns if the groups were in different persons. Furthermore, a calibrated clinical examiner assessed all clinical parameters, unaware of the group distribution, as much as possible. Moreover, males were selected to reduce the physiologic changes in hormone levels found in females that could exacerbate the gingival response to plaque. Smokers were excluded to minimize the interindividual variability in the gingival inflammatory response.[52]

GI was not assessed at days 11 and 18 so as not to interfere with plaque structure over established biofilm. We faced similar methodologic limitations with assessing the CGF volume on days 11 and 18. We had to remove supragingival plaque at the eight electronic gingival fluid measuring device sites before fluid collection to avoid plaque contamination of filter paper strips, assuring a minimum influence on volumetric determination of GCF.[50,53,54]

CHX was selected as the test substance because it is the best characterized and most effective chemical antiplaque agent.[38,41,44] The concentration selected (0.12%) corresponds to that used clinically for substitutive plaque control. The subjects' compliance during the experimental phase was 100%, as assured by the remaining CHX volume returned by them at the end of the experimental period.

As shown in Table 1, PI scores in the test group (initially plaque-covered surfaces) were statistically higher than in the control group (initially plaque-free surfaces) at all experimental periods, except baseline. However, we observed a distinct pattern of plaque deposition between test and control groups. In initially plaque-free surfaces, CHX did not stop plaque growth totally. This tendency of increasing plaque scores on initially plaque-free surfaces was expected as demonstrated in other studies.[38,43,55,56] However, after

day 11, initially plaque-covered surfaces showed a statistically significant decrease in plaque scores. This was an interesting finding that was not elucidated totally by the time frame of the study. However, one could hypothesize that when bacteria are located in a biofilm, the intermicrobial matrix could be affected by the CHX.[34] Thus, if the amount of intermicrobial matrix is diminished, a greater effect could be expected. Moreover, with different aims, Zaura-Arite et al.[35] demonstrated that 0.2% CHX gluconate promoted a superficial bacterial death in older biofilms. Other hypotheses for the observed decrease in PI in previously plaque-covered surfaces over time might be explained by the detachment of the external bacterial layers decreasing the mean PI. However, one interesting factor to investigate is whether this plaque accumulation is associated with different patterns of gingival inflammation.

The initially plaque-covered surfaces (test group) showed a significantly greater gingival inflammatory response, assessed by GI and GCF, compared to plaque-free surfaces (control group), except on day 0. Thus, we can assume that gingival inflammation was not different between the groups at baseline. These results suggested that the initial professional prophylaxis seemed to have an effect on preventing the occurrence of gingival inflammation in the absence of mechanical plaque disruption.

The importance of biofilm disruption prior to the initiation of a CHX regimen was investigated by Brownstein et al.[57] They compared the effects of rinsing with 0.12% CHX gluconate in sites with and without initial prophylaxis (split-mouth design) in individuals with preestablished gingivitis. CHX helped to decrease the mean GI, the percentage of sites with GI ≥2, and bleeding on probing only where initial prophylaxis had been performed. However, it must be emphasized that they studied the effects of CHX in reducing established gingivitis. In the present study, individuals started the experiment with gingival health, and the capacity of CHX to prevent gingivitis was tested.

Corbet et al.[58] tested the therapeutic effect of 0.12% CHX gluconate, compared to a placebo solution, in individuals with established untreated gingivitis in a parallel-control group design. The mean GI of the CHX group was reduced significantly from 1.40 to 1.08, with a marked reduction in the percentage of sites with GI = 2 (from 50% to 36%). This significant effect of CHX on gingivitis observed in the study may be too limited to ensure the prognostic benefits in the prevention of disease progression.

In our study, a statistically significant increase in GI was observed in plaque-free surfaces between days 0 and 25. Means ranged from 0.18 to 0.52. These results are in accordance with those from studies[37,56,59,60] that demonstrated a tendency for an increase in GI. However, those studies used the GI proposed by Löe and Silness,[61] whose score of 2 corresponds to bleeding upon periodontal probing of the external portion of the marginal gingival tissues. In our study, we used the GI modified by Löe,[51] whose score of 2 corresponds to bleeding upon periodontal probing of the intrasulcular marginal gingival tissues. Thus, the final mean GI could be higher because of the greater number of GI scores of 2. In addition, this method seems to be more adequate to the current knowledge diagnosis of gingivitis were bleeding after marginal periodontal probing is obtained in the internal gingival ulcerated epithelial due to the inflammatory response to biofilm stimuli.

The clinical relevance of this study indicates the necessity of previous removal of stablished biofilm when CHX needs to be used. Despite the fact that CHX is not commonly used in situations where the biofilm is already established, it is important to emphasize that this topic has gained focus with the studies of the interactions between oral microbiology and systemic diseases. For example, Joore et al.[62] recently suggested that oropharyngeal decontamination with either CHX or CHX/colistin reduced the development of ventilator-associated pneumonia (VAP). If it is demonstrated that the effect of CHX against accumulated plaque is marginal, mechanical tooth cleaning before CHX application might be a critical step in these cases.

It is acknowledged that the mode of action of CHX in plaque inhibition in vivo occurs via an immediate bactericidal effect, followed by prolonged bacteriostatic action resulting from its adsorption into the biofilm-coated enamel surface.[55,63] The hypothesis for the inefficacy of CHX over plaque-covered surfaces in preventing the establishment of gingivitis can be due the only superficial effect on mature biofilm where bacterial vitality remains in the internal part of biofilm.[36] Pratten et al.[34] exposed a biofilm, with a bacterial composition similar to that found in supragingival dental plaque, to 0.2% CHX gluconate solution; exposure for 1 or 5 minutes elicited no statistically significant effect on the viability of any of the six species tested.

Simulating intraoral biofilms, Zaura-Arite et al.[35] demonstrated, by means of confocal scanning laser microscopy, only a superficial bactericidal effect of CHX on in situ–grown dental biofilm. Auschill et al.,[64] in a similar study design, showed a mean bacterial viability reduction of 89% compared to water. In that study, CHX solution (0.2%) was applied intraorally for 1 minute twice a day for 4 days, and participants started to rinse at the same time that an in situ intraoral splint was inserted. Thus, only a thin bacterial layer was formed. The CHX-induced morphologic

alterations in oral biofilms were investigated by Vitkov et al.[33] A loss of bacterial membrane integrity and fimbrial disintegration were demonstrated in a few bacteria. A restricted matrix disintegration also was observed and showed the insufficient effect of CHX on oral biofilm. Kara et al.[65] recently showed that dual-species biofilms (*Streptococcus mutans* and *Veillonella parvula*) were less susceptible to CHX than single-species biofilms (*V. parvula*) because of the more favorable growth conditions generated by the additional lactate produced by *S. mutans*. These data showed that the environmental heterogeneity in multibacterial biofilms can accelerate phenotypic and genotypic diversity in bacterial populations, which might be the mechanisms whereby cells are better prepared to cope with adverse conditions, e.g., antimicrobial agents.

CONCLUSIONS

Initially plaque-free and plaque-covered surfaces showed increased plaque and gingival inflammation following abstinence from mechanical plaque control. However, the initially plaque-covered surfaces displayed greater amounts of plaque and gingival inflammation. Thus, professional removal of plaque is recommended before starting a CHX regimen as a plaque-control substitute.

ACKNOWLEDGEMENTS

The authors wish to thank the Lutheran University of Brazil, Canoas, RS, the Federal University of Rio Grande do Sul, Porto Alegre, RS, and the Franciscan University Center, Santa Maria, RS for the use of their clinics and the Periotron 8000 unit. Drs. Zanatta, Antoniazzi, and Rösing report no conflicts of interest related to this study.

REFERENCES

1. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol* 1995;49:711-745.
2. Bernimoulin JP. Recent concepts in plaque formation. *J Clin Periodontol* 2003;30:7-9.
3. Marsh PD. Dental plaque as a microbial biofilm. *Caries Res* 2004;38:204-211.
4. Marsh PD. Dental plaque: Biological significance of a biofilm and community life-style. *J Clin Periodontol* 2005;32:7-15.
5. Socransky SS, Haffajee AD. Dental biofilms: Difficult therapeutic targets. *Periodontol 2000* 2002;28:12-55.
6. Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000* 2005;38:135-187.
7. Albandar JM, Rams TE. Global epidemiology of periodontal diseases: An overview. *Periodontol 2000* 2002; 29:7-10.
8. Kinane DF, Attström R. European Workshop in Periodontology group B. Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European Workshop in Periodontology. *J Clin Periodontol* 2005;32:130-131.
9. Susin C, Oppermann RV, Haugejorden O, Albandar JM. Tooth loss and associated risk indicators in an adult urban population from south Brazil. *Acta Odontol Scand* 2005;63:85-93.
10. Löe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol* 1965;36:177-187.
11. Tatakis DN, Trombelli L. Modulation of clinical expression of plaque-induced gingivitis I. Background review and rationale. *J Clin Periodontol* 2004;31:229-238.
12. Trombelli L, Tatakis DN, Scapoli C, Bottega S, Orlandini E, Tosi M. Modulation of clinical expression of plaque-induced gingivitis II. Identification of "high-responder" and "low-responder" subjects. *J Clin Periodontol* 2004;31:239-252.
13. Trombelli L, Scapoli C, Orlandini E, Tosi M, Bottega S, Tatakis DN. Modulation of clinical expression of plaque-induced gingivitis. III. Response of "high responders" and "low responders" to therapy. *J Clin Periodontol* 2004;31:253-259.
14. Trombelli L, Scapoli C, Tatakis DN, Grassi L. Modulation of clinical expression of plaque-induced gingivitis: Effects of personality traits, social support and stress. *J Clin Periodontol* 2005;32:1143-1150.
15. Lovdal A, Arno A, Schei O, Waerhaug J. Combined effect of subgingival scaling and controlled oral hygiene on the incidence of gingivitis. *Acta Odontol Scand* 1961;19:537-555.
16. Axelsson P, Lindhe J. Effect of oral hygiene instruction and professional toothcleaning on caries and gingivitis in schoolchildren. *Community Dent Oral Epidemiol* 1981;9:251-255.
17. Rodrigues JA, dos Santos PA, Garcia PP, Corona SA, Loffredo LC. Evaluation of motivation methods used to obtain appropriate oral hygiene levels in schoolchildren. *Int J Dent Hyg* 2003;1:227-232.
18. Gjermo P, Baastad KL, Rölla G. The plaque inhibiting capacity of 11 antibacterial compounds. *J Periodontal Res* 1970;5:102-109.
19. Roberts WR, Addy M. Comparison of the in vivo and in vitro antibacterial properties of antiseptic mouthrinses containing chlorhexidine, alexidine, cetyl pyridinium chloride and hexetidine. Relevance to mode of action. *J Clin Periodontol* 1981;8:295-310.
20. Addy M. Chlorhexidine compared with other locally delivered antimicrobials. A short review. *J Clin Periodontol* 1986;13:957-964.
21. Fine DH. Chemical agents to prevent and regulate plaque development. *Periodontol 2000* 1995;8:87-107.
22. Tortora GJ, Funke BR, Case CL. Microbial development control. In: Tortora GJ, Funke BR, Case CL, eds. *Microbiologia*, vol. 6. Porto Alegre, Brazil: Artmed; 2000:181-206.
23. Rölla G, Melsen B. On the mechanism of the plaque inhibition by chlorhexidine. *J Dent Res* 1975;54:B57-B62.
24. Gjermo P, Bonesvoll P, Rölla G. Relationship between plaque-inhibiting effect and retention of chlorhexidine in the human oral cavity. *Arch Oral Biol* 1974;19: 1031-1034.
25. Bonesvoll P, Lökken P, Rölla G, Pous P. Retention of chlorhexidine in the human oral cavity after mouthrinses. *Arch Oral Biol* 1974;19:1025-1029.
26. Kozlovsky A, Artzi Z, Moses O, Kamin-Belsky N, Greenstein RB. Interaction of chlorhexidine with smooth and rough types of titanium surfaces. *J Periodontol* 2006;77:1194-1200.
27. Hjeljord LG, Rölla G, Bonesvoll P. Chlorhexidine-protein interactions. *J Periodontal Res Suppl* 1973;12:11-16.

28. Hugo WB, Longworth AR. Some aspects of the mode of action of chlorhexidine. *J Pharm Pharmacol* 1964; 16:655-662.
29. Dabrowska E, Letko M, Roszkowska-Jakimiec W, Letko R, Sadowski J. Effect of chlorhexidine mouthrinse on cathepsin C activity in human saliva. *Adv Med Sci* 2006;51(Suppl. 1):96-99.
30. Xie H, Cook GS, Costerton JW, Bruce G, Rose TM, Lamont RJ. Intergeneric communication in dental plaque biofilmes. *J Bacteriol* 2000;182:7067-7069.
31. Ghigo JM. Natural conjugative plasmids induce bacterial biofilm development. *Nature* 2001;412:442-445.
32. Donlan RM. Role of biofilms in antimicrobial resistance. *ASAIO J* 2000;46:S47-S52.
33. Vitkov L, Hermann A, Krautgartner WD, et al. Chlorhexidine-induced ultrastructural alterations in oral biofilm. *Microsc Res Tech* 2005;68:85-89.
34. Pratten J, Barnett P, Wilson M. Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria. *Appl Environ Microbiol* 1998;64:3515-3519.
35. Zaura-Arite E, Van Marle J, Tencate JM. Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *J Dent Res* 2001;80:1436-1440.
36. Decker EM, Weiger R, von Ohle C, Wiech I, Brecx M. Susceptibility of planktonic versus attached *Streptococcus sanguinis* cells to chlorhexidine. *Clin Oral Investig* 2003;7:98-102.
37. Löe H, Rindom Schiött C. The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. *J Periodontal Res* 1970;5:79-83.
38. Stoeken JE, Versteeg PA, Rosema NA, Timmerman MF, van der Velden U, van der Weijden GA. Inhibition of "de novo" plaque formation with 0.12% chlorhexidine spray compared to 0.2% spray and 0.2% chlorhexidine mouthwash. *J Periodontal* 2007;78:899-904.
39. Cumming BR, Löe H. Optimal dosage and method of delivering chlorhexidine solutions for the inhibition of dental plaque. *J Periodontal Res* 1973;8:57-62.
40. Brecx M. Strategies and agents in supragingival chemical plaque control. *Periodontolal 2000* 1997;15:100-108.
41. Hull PS. Chemical inhibition of plaque. *J Clin Periodontol* 1980;7:431-442.
42. Segreto VA, Collins EM, Beiswanger B, et al. A comparison of mouthrinses containing two concentrations of chlorhexidine. *J Periodontal Res* 1986(Suppl.); 21:23-32.
43. Smith RG, Moran J, Addy M, Doherty F, Newcombe RG, Smith RG. Comparative staining in vitro and plaque inhibitory properties in vivo of 0.12% and 0.2% chlorhexidine mouthrinses. *J Clin Periodontol* 1995;22:613-617.
44. Jones CG. Chlorhexidine: Is it still the gold standard? *Periodontol 2000* 1997;15:55-62.
45. Noiri Y, Okami Y, Narimatsu M, Takahashi Y, Kawahara T, Ebisu S. Effects of chlorhexidine, minocycline, and metronidazole on *Porphyromonas gingivalis* strain 381 in biofilms. *J Periodontol* 2003;74:1647-1651.
46. van der Weijden GA, Timmerman MF, Novotny AGA, Rosema NAM, Verkerk AAJ. Three different rinsing times and inhibition of plaque accumulation with chlorhexidine. *J Clin Periodontol* 2005;32:89-92.
47. Quigley GA, Hein JW. Comparative cleansing efficiency of manual and power brushing. *J Am Dent Assoc* 1962;65:26-29.
48. Turesky S, Gilmore ND, Glickman I. Reduced plaque formation by the chloromethyl analogue of victamine C. *J Periodontol* 1970;41:41-43.
49. Lang NP, Ostergaard E, Löe H. Fluorescent plaque disclosing agent. *J Periodontal Res* 1972;7:59-67.
50. Deinzer R, Mossanen BS, Herforth A. Methodological considerations in the assessment of gingival crevicular fluid volume. *J Clin Periodontol* 2000;27:481-488.
51. Löe H. The gingival index, the plaque index and the retention index systems. *J Periodontol* 1967;38:610-616.
52. Shearer B, Hall P, Clarke P, Marshall G, Kinane DF. Reducing variability and choosing ideal subjects for experimental gingivitis studies. *J Clin Periodontol* 2005;32:784-788.
53. Stoller NH, Karras DC, Johnson LR. Reliability of crevicular fluid measurements taken in the presence of supragingival plaque. *J Periodontol* 1990;61:670-673.
54. Griffiths GS, Wilton JMA, Curtis MA. Contamination of human gingival crevicular fluid by plaque and saliva. *Arch Oral Biol* 1992;37:559-564.
55. Addy M, Moran J. Comparison of plaque accumulation after topical application and mouth rinsing with chlorhexidine gluconate. *J Clin Periodontol* 1983;10:69-71.
56. Löe H, Schiött CR, Karring G, Karring T. Two years oral use of chlorhexidine in man. I. General design and clinical effects. *J Periodontal Res* 1976;11:135-144.
57. Brownstein CN, Briggs SD, Schweitzer KL, Briner WW, Kornman KS. Irrigation with chlorhexidine to resolve naturally occurring gingivitis. A methodologic study. *J Clin Periodontol* 1990;17:588-593.
58. Corbet EF, Tam JO, Zee KY, et al. Therapeutic effects of supervised chlorhexidine mouthrinses on untreated gingivitis. *Oral Dis* 1997;3:9-18.
59. Ramberg P, Furuichi Y, Volpe AR, Gaffar A, Lindhe J. The effects of antimicrobial mouthrinses on de novo plaque formation at sites with healthy and inflamed gingivae. *J Clin Periodontol* 1996;23:7-11.
60. Waerhaug M, Gjermo P, Rölla G, Johansen JR. Comparison of the effect of chlorhexidine and $CuSO_4$ on the plaque formation and development of gingivitis. *J Clin Periodontol* 1984;11:176-180.
61. Löe H, Silness J. Periodontal disease in pregnancy I. Prevalence and severity. *Acta Odontol Scand* 1963; 21:533-551.
62. Koeman M, van der Ven AJ, Hak E, et al. Oral decontamination with chlorhexidine reduces the incidence of ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2006;173:1348-1355.
63. Jenkins S, Addy M, Wade W. The mechanism of action of chlorhexidine. A study of plaque growth on enamel inserts in vivo. *J Clin Periodontol* 1988;15:415-424.
64. Auschill TM, Hein N, Hellwig E, Follo M, Sculean A, Arweiler NB. Effect of two antimicrobial agents on early in situ biofilm formation. *J Clin Periodontol* 2005; 32:147-152.
65. Kara D, Luppens SB, Cate JM. Differences between single- and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and susceptibility to chlorhexidine. *Eur J Oral Sci* 2006;114:58-63.

Correspondence: Dr. Fabrício B. Zanatta, Rua Humberto de Campos, 250/302, 97095-230, Santa Maria, RS, Brazil. Fax: 55-51-33468789; e-mail: fabriciobzanatta@yahoo.com.br.

Submitted February 9, 2007; accepted for publication May 3, 2007.