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ORIGINAL ARTICLE

In vivo substantivity of 0.12% and 0.2% chlorhexidine mouthrinses on salivary bacteria

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Abstract The in vivo antimicrobial activity of 0.12% and 0.2% chlorhexidine (CHX) on the salivary flora up to 7 h after its application, using epifluorescence microscopy with the SYTO 9/propidium iodide dual staining, was evaluated. Fifteen volunteers performed a single mouthrinse with sterile water (SM-water), a single mouthrinse with 0.12% CHX (0.12% SM-CHX) and a single and double mouthrinse with 0.2% CHX (0.2% SM-CHX and 0.2% DM-CHX). Samples of saliva were taken at 30 s, and 1, 3, 5, and 7 h after each application. In comparison with SM-water, 0.2% CHX (SM and DM) showed a significant antibacterial effect up to 7 h after the mouthrinse, whereas this effect only persisted up to 5 h after the 0.12% SM-CHX mouthrinse. On comparing the two concentrations of CHX, significantly higher percentages of bacterial vitality were observed in all the saliva samples after the use of 0.12% CHX than after 0.2% CHX. On comparison of the 0.2% SM-CHX and 0.2% DM-CHX, significantly higher percentages of live bacteria were observed in the saliva samples taken at 1, 3, 5, and 7 h after the single mouthrinse compared with the double mouthrinse. The 0.2% CHX mouthrinse had the greatest antimicrobial activity on the salivary flora up to 7 h after its application, with a progressive recovery in bacterial vitality. The differences observed with respect to the 0.12% CHX mouthrinse demonstrate the influence of the concentration on its immediate antimicrobial activity and substantivity.

Keywords Antibacterial activity · Chlorhexidine · Concentration · Epifluorescence microscopy · Saliva · Substantivity

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Introduction

The study of the in vivo antibacterial activity of an antiseptic involves the analysis of its immediate effect and of its substantivity. Substantivity is defined as the prolonged adherence of the antiseptic to the oral surfaces (teeth and mucosas) and its slow release at effective doses that guarantees the persistence of its antimicrobial activity [1]. Numerous authors have showed that CHX has a greater in vivo immediate antibacterial effect and a greater substantivity than other antiseptics used in the oral cavity [2–5].

For some decades now, the determination of salivary bacterial counts has been a test accepted by the scientific community to investigate the in vivo antibacterial effect of CHX [6, 7] and is considered to be predictive of its substantivity [3, 8, 9] and of its potential antiplaque activity [10]. Since the first results were reported by Schiott et al in 1970 [11], numerous studies evaluating the substantivity of CHX on the salivary flora have been published [2–5]. However, few authors have studied the in vivo antibacterial effect and the duration of this effect after a single application of 0.2% CHX compared with 0.12% CHX on the salivary flora; rather, they have mainly analyzed the influence of the dose administered (by applying different volumes of each concentration) [12, 13].

In the majority of series published, the determination of the antimicrobial activity of CHX in saliva was performed using plate culture microbiological techniques [2–5, 14]. However, some authors have questioned the reliability of these methods and, as an alternative, have proposed the use of fluorescent methods that use specific fluorochromes to mark live and dead bacteria [15, 16]. The objective of this study was to evaluate the in vivo antimicrobial activity of a 0.2% and 0.12% CHX digluconate mouthrinse on the salivary flora up to 7 h after its application, using the epifluorescence microscopy with the SYTO 9/propidium iodide dual staining.

Material and methods

- Selection of the study group

The study group was composed of 15 adult volunteers between 20 and 45 years of age, and who presented a good oral health status: minimum of 24 evaluable permanent teeth with no evidence of gingivitis or periodontitis (Community Periodontal Index score=0) [17] and an absence of caries. The following exclusion criteria were applied: smoker, presence of dental protheses or orthodontic devices, antibiotic treatment or the routine use of oral antiseptics during the previous 3 months, and the presence of any systemic disease that could lead to an alteration in the production and/or composition of the saliva. A professional tooth cleaning was performed on all volunteers before entering the study.

Non-stimulated samples of saliva (1 mL) were collected from each patient under basal conditions and at 30 s and 1, 3, 5, and 7 h after performing the following mouthrinse under supervision:

1) A single, 30-s mouthrinse with 10 mL of sterile water (negative control; SM-water).
2) A single, 30-s mouthrinse with 10 mL of 0.12% CHX (Paroex®; DistriFarma, Barcarena, Portugal; 0.12% SM-CHX).
3) A single, 30-s mouthrinse with 10 mL of 0.2% CHX (Oraldine Perio®; Pfizer, Barcelona, Spain; 0.2% SM-CHX).
4) A double, (two consecutives), 30-s mouthrinse with 10 mL of 0.2% CHX (Oraldine Perio®; Pfizer, Barcelona, Spain; 0.2% DM-CHX).

The volunteers were not allowed to practice any oral hygiene technique from the previous midnight. In the experiment day, the time of sample collection ranged from 11:50 a.m. (baseline sample) to 7 p.m. (last sample collected 7 h after ending mouthrinse). The volunteers were not allowed to eat or drink anything for 1 h prior to the collection and during the course of the experiment. The non-stimulated saliva samples were collected using the spitting method [18]. Using a system of balanced randomization, all volunteers performed the four mouthrinses with a washout period of 2 weeks between each test. The project was approved by the Ethics Committee of the Faculty of Medicine and Dentistry of Santiago de Compostela University. Written informed consent was obtained from all the participants in the study.

- Processing of the saliva samples

The SYTO 9/propidium iodide (PI) dual fluorescence staining (LIVE/DEAD® BacLight™) was prepared following the manufacturer's recommendations in 5 ml of sterile-filtered water using a 0.22-μm Millipore membrane filter (Millipore Ibérica S.A., Madrid, Spain), with a 1:1 ratio of both fluorochromes, and was stored at −20ºC. The saliva samples were centrifuged at 2,000 rpm for 6 min. The supernatant was discarded, and the pellet obtained was resuspended in 100 μL of sterile water. After homogeneizing the bacterial suspension by shaking, it was mixed with 100 μL of the fluorescence solution and was stored in the dark at room temperature for 15 min. Observations were performed by two researchers, who did not know the study design, using an Olympus BX51 microscope (Olympus, Tokyo, Japan) fitted with an Olympus DP70 camera and a set of filters for fluorescein and Texas Red. The count of live and dead bacteria was performed at high magnification (×100) on 20 microscope fields that presented a minimum of 100 bacteria (bacterial aggregates were excluded). The mean percentage of live bacteria was calculated for each saliva sample, and the difference in the percentage of live bacteria between two saliva samples was called the "vitality reduction" (VR). Positive values represent a decrease in bacterial vitality, and negative values means an increase in bacterial vitality.

Statistical analysis

The results were analyzed using the SPSS version 15.0 statistical package for Windows (SPSS Inc., Chicago, Illinois, USA). The intraclass correlation coefficient (ICC) test was used for the analysis of intra-observer and inter-observer correlations. The repeated measure ANOVA test was used for intra-mouthrinse and inter-mouthrinse comparisons using all the saliva samples, and simple and repeat comparisons for the analysis of intra-mouthrinse and inter-mouthrinse comparisons between two saliva samples. Statistical significance was taken as a P value less than 0.05.

Results

In the intra-observer analysis, the ICC mean value was 0.90 ($P<0.001$), and in the inter-observer analysis, the ICC mean value was 0.92 ($P<0.001$).

Figure 1 shows the mean percentages of bacterial vitality in saliva under basal conditions and at 30 s and 1, 3, 5, and 7 h after the mouthrinses with SM-water, 0.12% SM-CHX, 0.2% SM-CHX, and 0.2% DM-CHX.

Statistically significant differences in bacterial vitality were detected in the intra-mouthrinse analyses between the different sample collection times ($P<0.001$; Table 1). In comparison with the baseline values, the frequency of live bacteria decreased significantly at 30 s after the SM-water (VR=10.13±0.51, $P<0.001$), 0.12% SM-CHX (VR=86.77±6.36, $P<0.001$), 0.2% SM-CHX (VR=91.35±4.37, $P<0.001$), and 0.2% DM-CHX (VR=89.41±10.70, $P<0.001$). In comparison with the baseline values, 0.2% CHX (SM and DM) presented significant antibacterial activity up to 7 h after the mouthrinse (VR=14.14±11.56 and 39.16±19.35, respectively, $P<0.001$ for both mouthrinses), whereas this activity only persisted for 3 h after application of the 0.12% CHX (VR=19.88±20.07, $P<0.05$). In comparison with the values obtained 30 s after the mouthrinse, a significant recovery of the bacterial population was observed in the later saliva samples taken after the different mouthrinses; 0.12% SM-CHX presented the highest percentages of recovery, and 0.2% DM-CHX presented the lowest percentages (Table 1).

In the inter-mouthrinse analysis, statistically significant differences were detected in the percentage of live bacteria between the different types of mouthrinse ($P<0.001$; Table 2). In comparison with SM-water, the prevalence of live bacteria was significantly lower at 30 s after the mouthrinses with 0.12% CHX (VR=75.06±9.89, $P<0.001$) and with 0.2% CHX (VR of the SM=81.93±2.46, $P<0.001$; VR of the DM=82.28±1.89, $P<0.001$). In comparison with SM-water, 0.2% CHX (SM and DM) showed a significant antibacterial effect up to 7 h after the mouthrinse (VR=16.26±11.72 and 38.21±22.54, respectively; $P<0.001$ for both mouthrinses), whereas this effect only persisted up to 5 h after the 0.12% SM-CHX mouthrinse (VR=7.66±8.64, $P<0.05$). On comparing the two concentrations of CHX, significantly higher percentages of bacterial vitality were observed in all the saliva samples after the use of 0.12% CHX than after 0.2% CHX, varying from a VR at 30 s of 6.86±9.34 ($P<0.05$) to a VR at 3 h of 41.93±16.22 ($P<0.001$). On comparison of the 0.2% SM-CHX and 0.2% DM-CHX, significantly higher percentages of live bacteria were observed in all the saliva samples taken after 1 h after the use of the single mouthrinse compared with the double mouthrinse, with the VR ranging from 4.85±6.50 ($P<0.05$) at 1 h to 22.78±19.54 ($P<0.001$) at 7 h (Table 2).

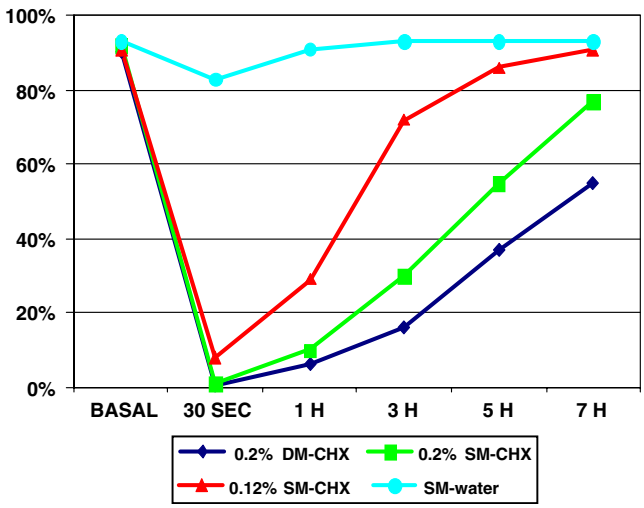

Fig. 1 Percentages of bacterial vitality in saliva under basal conditions and at 30 s and 1, 3, 5, and 7 h after the application of sterile water (single mouthrinse), 0.12% chlorhexidine (single mouthrinse), and 0.2% chlorhexidine (single and double mouthrinse). *SM* single mouthrinse, *DM* double mouthrinse, *CHX* chlorhexidine. *Basal* saliva sample collected under basal conditions, *30 s* saliva sample collected at 30 s after the application of the different mouthrinses, *1 h* saliva sample collected 1 h after the application of the different mouthrinses, *3 h* saliva sample collected 3 h after the application of the different mouthrinses, *5 h* saliva sample collected 5 h after the application of the different mouthrinses, *7 h* saliva sample collected 7 h after the application of the different mouthrinses

Discussion

Recently, our group showed that epifluorescence microscopy with the SYTO 9/PI dual staining is an efficient method that permits the evaluation of the antimicrobial activity of CHX on the salivary flora in real time [19]. Since the epifluorescence microscopy has been considered an observer-dependent technique [19], it is important to determine the intra-observer and inter-observer correlations. In the present series, the ICC mean values were more than or 0.90. To date, we have not found other studies where the SYTO 9/PI solution has been used to evaluate the in vivo CHX activity on salivary flora; the comparison of our results with those obtained by other authors using plate culture techniques should therefore be interpreted with caution.

Using epifluorescence microscopy and the DF/BE solution, Weiger et al [15] found that approximately 85% of the salivary flora is live under basal conditions. These results agree with those obtained previously by our group using the SYTO 9/PI solution [16] and with the results obtained in the present series (vitality varied between 90–93%).

In agreement with the results obtained by some authors using plate culture techniques [20, 21], the single mouthrinse with sterile water lead to a significant decrease in bacterial vitality (10%) at 30 s after the mouthrinse in the present series, although this decrease was transitory.

Many authors have also detected the immediate antibacterial effect and the persistence of its substantivity for a minimum of 7 h after a mouthrinse with 0.12% SM-CHX

Table 1 Intra-treatment comparisons of the percentage of bacterial vitality in saliva under basal conditions compared with samples taken at 30 s and at 1, 3, 5, and 7 h after the application of sterile water (single mouthrinse), 0.12% chlorhexidine (single mouthrinse), and 0.2% chlorhexidine (single and double mouthrinse)

Mean difference±standard deviation (%)

	SM-water	0.12% SM-CHX	0.2% SM-CHX	0.2% DM-CHX
Basal[a] vs 30 s[b]	10.13±0.51**	86.77±6.36**	91.35±4.37**	89.41±10.70**
Basal[a] vs 1 h[c]	0.60±1.29	68.33±17.99**	81.92±8.66**	84.25±10.93**
30 s[b] vs 1 h[c]	−9.53±1.18**	−21.33±17.77**	−9.53±6.24**	−5.28±4.14**
Basal[a] vs 3 h[d]	0.26±1.48	19.88±20.07*	61.35±15.09**	77.00±9.78**
30 s[b] vs 3 h[d]	−9.86±1.45**	−64.00±16.44**	−28.93±14.10**	−15.35±10.78**
Basal[a] vs 5 h[e]	−0.40±1.68	3.33±11.29	35.50±18.77**	54.66±22.61**
30 s[b] vs 5 h[e]	−10.53±1.76**	−77.93±14.55**	−54.33±19.21**	−37.07±18.37**
Basal[a] vs 7 h[f]	−0.26±0.79	−1.55±10.15	14.14±11.56**	39.16±19.35**
30 s[b] vs 7 h[f]	−10.40±0.91**	−83.13±12.42**	−76.06±11.72**	−54.50±22.13**

Positive values represent a decrease in bacterial vitality and negative values means an increase in bacterial vitality.

SM single mouthrinse

DM double mouthrinse

CHX chlorhexidine

*$P<0.05$; **$P<0.001$

[a] Saliva sample collected under basal conditions

[b] Saliva sample collected at 30 s after the application of the different mouthrinses

[c] Saliva sample collected 1 h after the application of the different mouthrinses

[d] Saliva sample collected 3 h after the application of the different mouthrinses

[e] Saliva sample collected 5 h after the application of the different mouthrinses

[f] Saliva sample collected 7 h after the application of the different mouthrinses

Table 2 Inter-mouthrinse comparisons of the percentage of bacterial vitality in saliva under basal conditions and in the post-mouthrinse samples collected at 30 s and 1, 3, 5, and 7 h after the application of sterile water (single mouthrinse), 0.12% chlorhexidine (single mouthrinse), and 0.2% chlorhexidine (single and double mouthrinse)

Mean difference±standard deviation (%)

	Basal[a]	30s[b]	1h[c]	3h[d]	5h[e]	7h[f]
SM-water vs 0.12% SM-CHX	1.55±7.02	75.06±9.89**	63.26±15.22**	20.93±14.83**	7.66±8.64*	2.33±6.35
SM-water vs 0.2% SM-CHX	0.85±5.49	81.93±2.46**	81.93±7.95**	62.86±15.32**	38.13±19.80**	16.26±11.72**
SM-water vs 0.2% DM-CHX	3.00±10.74	82.28±1.89**	86.64 ±4.05**	76.92±10.99**	55.92±18.58**	38.21±22.54**
0.12% SM-CHX vs 0.2% SM-CHX	−2.87±9.09	6.86±9.34*	18.66±17.67**	41.93±16.22**	30.46±17.97**	13.93±11.16**
0.2% SM-CHX vs 0.2% DM-CHX	−0.09±5.02	0.42±1.65	4.85±6.50*	13.57±13.58*	19.00±23.83*	22.78±19.54**
0.12% SM-CHX vs 0.2% DM-CHX	2.66±18.80	7.14±9.67*	23.71±15.98**	56.28±13.96**	48.42±17.48**	36.64±21.73**

Positive values represent a decrease in bacterial vitality and negative values means an increase in bacterial vitality.

SM single mouthrinse

DM double mouthrinse

CHX chlorhexidine

*$P<0.05$; **$P<0.001$

[a] Saliva sample collected under basal conditions

[b] Saliva sample collected at 30 s after the application of the different mouthrinses

[c] Saliva sample collected 1 h after the application of the different mouthrinses

[d] Saliva sample collected 3 h after the application of the different mouthrinses

[e] Saliva sample collected 5 h after the application of the different mouthrinses

[f] Saliva sample collected 7 h after the application of the different mouthrinses

(15 mL/30 s), although the immediate decrease in vitality in this case did not exceed 72–87% (<1 $\log_{10}$) [22–25] and was 58–88% (<1 $\log_{10}$) at 7 h after the mouthrinse [4, 22, 25]. In the present series, 0.12% SM-CHX produced an immediate decrease in the percentage of live bacteria of less than 90% compared with baseline values, and this antimicrobial activity was only detectable up to 3 h after the mouthrinse, at which point, the reduction in vitality was of 20%.

Other authors have demonstrated that the application of 0.2% SM-CHX (10 mL/1 min) was associated with an immediate antibacterial effect with a reduction of more than or 90% (≥1 $\log_{10}$) in the bacterial concentration with respect to the baseline values [2, 26–29] and its substantivity persisted for a minimum of up to 7 hours after the mouthrinse, with a reduction of more than or 90% [2, 26, 27, 29]. In the present series, the mouthrinses with 0.2% CHX (SM and DM) resulted in an immediate decrease in the percentage of live bacteria (≥90%), and this antimicrobial activity was still detectable 7 h after the mouthrinse, with reductions in vitality of 14% (SM) and 39% (DM) compared with baseline values.

Despite the importance given to the concentration of CHX with respect to its antimicrobial activity [30], we have not found any study that has evaluated in vivo the influence of CHX concentration (0.2% vs 0.12%) on its antibacterial activity on the salivary flora. Recently, using plate culture techniques, our group observed that a mouthrinse with 0.2% CHX (10 mL/30 s) was associated with an immediate antimicrobial activity that was maintained for 1 h after its application and that this was significantly greater than the effect of a mouthrinse with 0.12% CHX (10 mL/30 s) [14]. In the present series, 0.2% CHX produced a significantly greater antibacterial effect than 0.12% CHX in all the saliva samples obtained after the mouthrinse. These results confirm that the antibacterial effect of CHX and its substantivity are concentration dependent, as previously reported by other authors who compared different concentrations of CHX (0.12% vs 0.06%, 0.06% vs 0.03%, and 0.12% vs 0.1%) [12, 31].

Dahlen [32] observed that a double mouthrinse with 0.2% CHX (10 mL/1 min each mouthrinse) produced a greater antibacterial effect at 10 min and 1 h after the mouthrinse than a single mouthrinse with 0.2%. In the present series, the 0.2% DM-CHX produced a significantly greater antibacterial effect than the 0.2% SM-CHX mouthrinse on the saliva samples collected at 1, 3, 5, and 7 h after the mouthrinse. This finding suggests that not only concentration but also other variables such as mouthrinse dose or duration may also condition CHX substantivity.

Many authors have observed that the immediate antibacterial effect of a single application of 0.2% or 0.12% CHX remained constant for a minimum of up to 7 h after the mouthrinse [2, 12, 24, 26, 27, 29, 33]. However, other authors have detected a continued fall in bacterial vitality up to 3–5 h after the mouthrinse, with a subsequent recovery of the salivary flora at 7 h after the mouthrinse [3, 34]. In accordance with the results reported in some in vitro studies [35], in the present series, a significant recovery in bacterial vitality was detected in all the saliva samples with the three different CHX mouthrinses in comparison with the vitality at 30 s after the mouthrinse. These differences could be due to methodological variations and highlight the need to clarify the influence of CHX substantivity on the recovery of the salivary flora after its application.

In conclusion, the 0.2% CHX mouthrinse had the greatest antimicrobial activity on the salivary flora up to 7 h after its application, with a progressive recovery in bacterial vitality. The differences observed with respect to the 0.12% CHX mouthrinse demonstrate the influence of the concentration on its immediate antimicrobial activity and substantivity.

These results may help to optimize antiseptic protocols in clinical situations where a significant decrease of bacterial load during short time is required as the prevention of post-operative infections.

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Conflict of interest The authors declare that they have no conflict of interest.

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