Original Article

Comparison of the Substantivity of Several Mouthwashesand their Effect on Microbial Plaque using Epifluorescence Microscope

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Abstract

Background and Aim: Several techniques have been used to assess the efficacy and substantivity of mouthwashes. Considering the variability of available mouth rinses, this study aimed to assess the substantivity of several mouthwashes and their effect on microbial plaque using an epifluorescence microscope.

Materials and Methods: This crossover double blind clinical trial evaluated 0.2%, and 0.12% (from 2 different brands) chlorhexidine (CHX), Persica and cetyl pyridinium chloride (CPC) mouth washes as well as normal saline as the negative control. Non-stimulated saliva samples were obtained from 16 candidates at baseline, 30s, 1, 3, 5 and 7h after one time use of mouthwashes. Epifluorescence technique was used to assess the viability of bacteria. For clinical examination following prophylaxis, subjects were asked not to use any oral hygiene measure except for the provided mouthwashes (twice a day) for 4 days. A 10-day washout period was allowed between the use of mouthwashes. Repeated measures ANOVA and Scheffe's test were applied for the comparison of viable bacterial count between the groups and Kruskal Wallis test was used for the assessment of microbial plaque.

Results: Persica and 0.12% CHX maintained their substantivity for 3 and 5h, respectively. A significant reduction in bacterial count was observed up to 7h after the use of 0.2% CHX only ($p<0.001$). On clinical examination, 0.2% and 0.12% CHX mouthwashes had significant differences with the others but had no significant difference with one another ($p<0.02$). Persica and CPC had similar efficacy ($p<0.02$).

Conclusion: Efficacy of mouthwashes strongly depends on their substantivity. Daily application frequency of other mouth rinses should be increased in order to achieve an efficacy equal to that of CHX.

Key Words: Chlorhexidine, Cetyl pyridinium chloride, Saliva, Epifluorescence microscope, Substantivity

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Introduction

Frequent and precise removal of microbial plaque is the mainstay of prevention and treatment of almost all types of chronic periodontal diseases. Use of effective and safe adjuncts to supplement mechanical plaque removal is also indicated for plaque control [1, 2]. Mouthwashes are among the most effective antimicrobial agents for chemical plaque control. Different chemicals have been introduced to the market for use as mouthwash. Most studies have focused on CHX and CPC solutions. CHX is considered as the gold standard in this respect [3]. Efficacy of CHX is due to its high substantivity in the oral environment and its high antimicrobial activity [4]. Substantivity of a mouthwash refers to its ability to maintain antibacterial

properties in the oral environment. The substantivity of antimicrobial agents in the oral environment is an important parameter for prevention of microbial plaque growth [5]. In the recent years, CHX has been manufactured in variable concentrations with claims of higher efficacy.

Another substance becoming increasingly popular as a mouth rinse is Salvadora Persica or toothbrush tree extract. Its use by Middle Easterners goes back to 1400 years ago [6].

Several techniques are available for the assessment of the efficacy of mouth rinses. The majority of previous studies have clinically investigated the effect of mouthwashes on prevention or reduction of microbial plaque [7-9]. Berchier et al. stated that 0.2% CHX had a significantly greater effect than 0.12% CHX on plaque control [9]. Versterg et al. reported that the efficacy of 0.07% CPC-containing solution was equal to that of 0.1% CHX for prevention of plaque growth [10]. Another study showed that CHX was 2.5 times more effective than Persica for plaque elimination [11]. In the recent years, for the assessment of substantivity of mouthwashes, their effects on oral microbial flora have been investigated using microbial culture or fluorescence techniques [12, 13]. The fluorescence technique is newer and only a limited number of studies have used this technique. It is more affordable than the culture technique and is less time-consuming [12]. Tomas et al. used this technique and showed that the antibacterial activity of 0.2% CHX was greater than that of 0.12% CHX [12]. In another study, Tomas et al. demonstrated that the percentage of viable bacteria decreased after the use of 0.2% CHX compared to their baseline count [13]. In the review of literature, we could not find any study on the efficacy and substantivity of different mouthwashes in the laboratory and clinical settings, simultaneously. Considering the variability of mouth rinses available in the market, this study aimed to compare the substantivity of several mouthwashes and their effect on microbial plaque using an epifluorescence microscope.

Materials and Methods

This double blind crossover clinical trial was conducted on subjects who met the following inclusion criteria: having a minimum of 24 teeth, not using fixed or removable orthodontic appliances or fixed or removable partial dentures, not having a sulcus depth over 3mm, having a maximum score of 2 ofTuresky-Modified Quigley-Hein Plaque Index [14] and Loe and Silness gingival index [15] maximum score of 1.5.

The exclusion criteria were smoking, antibiotic therapy, regular use of an oral antiseptic during the past 3 months, systemic disease affecting the composition of saliva, allergy to CHX and loss to follow up. All periodontal examinations were performed by a periodontist. The study design was approved by the Ethics Committee and Research Council of Islamic Azad University, Dental School and registered in the clinical trial registry system (IRCT201204289582N1). Based on the results of a similar study [13] and using the comparison option of Minitab software, the minimum required sample size was calculated to be 16 subjects taking into account α=0.05 and β= 0.2. The study was thoroughly explained to subjects and written informed consent was obtained from them. A form was also completed including demographic characteristics of patients and data regarding the study criteria. The mouthwashes evaluated in this study were as follows:

1)0.2% CHX mouthwash (SHAHR DAROU LABORATORIES, Iran)

2)0.12% CHX mouthwash (DonyayeBehdasht Co.,Iran)

3)0.12% CHX mouthwash (LivarPharmaceutical Lab., Spain)

4)0.05PC mouthwash (Oral-B, USA)

5)Persica mouthwash (Sourmaghy,Iran)

6)Normal saline (negative control)

All understudy subjects used all the understudy mouthwashes in a crossover design with 10-day washout periods.

In the first step, substantivity of mouth rinses was evaluated. In each treatment course, non-stimulatedsaliva samples were collected at baseline, 30s, 1, 3, 5 and 7h after one time use of 10ml of the mouthwash for 30s. Instructions on the method and frequency of the use of mouthwashes were written in a piece of paper by a person other than the researchers, placed in coded envelopes and given to participants. The investigator (periodontist) was blinded to the envelope contents (single blind). Participants were asked not to use any oral hygiene measure from the midnight before

the sampling. Also, they were requested not to smoke, drink or eat from an hour before and during the saliva sampling. Salivary samples were collected by spitting into sterile tubes and transferred to the Milad Hospital Laboratory within 2 hours after collection. In the lab, specimens were frozen for long-term storage at -80°C. The bacterial viability kit was opened. Live/Dead BacLight Fluorescence solution (Invitrogen, USA) was used according to the manufacturer's instructions. After using the staining agent, the dyestainsboth live and dead bacteria as green and red under the microscope. The laboratory technician was unaware of the type of specimen (double blind).

In the next step, the anti-plaque efficacy of mouthwashes was evaluated in the understudy participants.

In the first visit after data registry, the baseline plaque index of subjects (not using any oral hygiene measure for the past 12-18h) was measured using Turesky-Modified Quigley-Hein Plaque Index [14]. All clinical examinations were made by a periodontist blinded to the treatment plan and type of mouthwash used. After prophylaxis with ultrasonic or hand instruments, participants were requested to use the selected mouthwash twice a day as instructed. After 4 days, the plaque index of participants not using any oral hygiene measure for the past 12-18h was measured using Turesky-Modified Quigley-Hein Plaque Index [14].

After completion of the period of usage of each mouthwash, a 10-day washout period was allowed and then the next mouthwash was prescribed for use in the same manner (cross-over design). The incidence of side effects namely burning sensation and change in the sense of taste was also assessed using a 1-10 subjective scale.

The viable bacterial count in groups was assessed using repeated measures ANOVA. Scheffe's post-hoc test was applied for inter-group comparisons. Kruskal Wallis test was used for microbial plaque assessment. Data were analyzed using SPSS version software.

Results

The This study was performed on 16 subjects including 12 females (75%) with a mean age of 24.25±1.2 yrs. and 4 males (25%) with a mean age of 28± 3.6 yrs. (range 22-32 yrs.). Number of teeth was 28.3±2.5 in men and 28.4±1.6 in women (range 26-32 teeth). Seven cases had crowding; which was in the anterior mandible in 5 and in the anterior segment of both jaws in 2 cases.

Table 1 shows the degree of substantivity or number of viable bacteria in the understudy subjects at different time points based on the type of mouthwash used. As seen in Table 1, number of viable bacteria was the same at baseline and had no statistically significant difference ($p<0.8$).

At 30s, repeated measures ANOVA showed a significant difference among the 6 groups ($p<0.001$). Scheffe's post-hoc test demonstrated that the difference between the normal saline negative control group and the remaining 5 groups was statistically significant ($p<0.01$) and the lowest viable bacterial count belonged to 0.2% CHX followed by 0.12% CHX and Livarthat had significant differences with CPC and Persica ($p<0.01$). However, CPC and Persica were similar in this regard.

At one hour, similar results to 30s were obtained.

At 3h, repeated measures ANOVA showed that the lowest viable bacterial count belonged to 0.2% CHX while the highest count belonged to normal saline ($p<0.001$). Comparison of 5 mouthwashes by repeated measures ANOVA showed that the viable bacterial count was the lowest in 0.2% CHX and the highest in Persica ($p<0.01$). Multiple comparisons showed that this difference was related to the difference of 0.2% CHX mouthwash with the four other mouth rinses and no significant difference was found between the remaining 4 mouthwashes ($p<0.3$). Also, at this time point, Persica was no longer substantive and number of viable bacteria had reached their baseline count. However, other mouth rinses were still substantive.

At 5h, repeated measures ANOVA revealed that the viable bacterial count was significantly different in the 6 groups ($p<0.001$); excluding the Persica and negative control groups, the difference between the remaining 4 groups was not significant. Since Persica did not have substantivity at 3h follow up, it was not compared with the remaining groups at 5h. Based on therepeated measures ANOVA, number of viable bacterial count was significantly different between the 4 mouthwash groups ($p<0.001$) and pairwise comparison showed that this difference was only due to the difference of 0.2% CHX with the remaining 3 groups and the

Table 1. Viable bacterial count in the understudy groups at different times

	Baseline	30s	1h	3h	5h	7h
0.2% CHX	359/8±98/6	40/06±21/1	71/2±22/1	87/1±20/18	98/8±15/6	170/6±18/3
0.12% CHX	333/7±89/9	43/6±16/9	79/8±20/2	145/6±44/8	166/6±41/4	304/5±109/3
Livar	358/3±168/3	45/06±20/4	85/7±32/02	149/3±72/7	179/7±91/9	295/5±118/6
CPC	350/5±110/9	70/06±31/4	98/1±49/4	124/3±43/5	187/3±45/3	314/1±118/7
Persica	363/6±176/3	67/1±19/1	107/3±45/5	283/9±154/8	307/6±173	370/4±154/5
Saline	351/7±110/8	289/2±199/5	302/2±105/5	338±112/9	345/8±226/2	359/8±202/3

remaining 3 were not significantly different ($p<0.3$). At this time point, all 4 remaining mouth rinses maintained their substantivity and number of viable bacterial count had not returned to the baseline value ($p<0.001$).

At 7h, repeated measures ANOVA showed a statistically significant difference between groups ($p<0.001$). Pairwise comparison of groups revealed that this difference was significant only between the 0.2% CHX and the remaining 5 groups and no significant difference was found between the remaining 5 groups. At this time point, only 0.2% CHX still maintained its substantivity and number of viable bacterial count was still lower than baseline ($p<0.001$).

The plaque regrowth in the 4 mouthwash groups after 4 days is shown in Table 2 indicating that the lowest plaque regrowth (1.57±0.21) belonged to the 0.2% Iranian CHX and the highest belonged to normal saline (3.30±0.54). Kruskal Wallis test showed that this difference was statistically significant ($p<0.001$). Except for normal saline, comparison of the 5 groups revealed that 0.2% and 0.12% CHX had significant differences with the remaining 2 mouthwashes but did not have significant differences with one another ($p<0.02$). Also, Persica and CPC had similar efficacy ($p<0.02$).

Table 2. The 4-day plaque index of subjects in different groups

Mouthwash/Plaque regrowth	Plaque index	P value
CPC (Oral-B)	2/39±0/45	0/001
0.12% CHX (Livar)	1/41±0/4	
0.2% CHX (Iranian)	1/57±0/21	
0.12% CHX (Iranian)	1/65±0/4	
Persica	1/99±0/14	
Saline	3/30±0/54	

Evaluation of side effects of mouthwashes showed that the highest frequency of burning sensation belonged to the Iranian 0.2% CHX (4±1.9) and the lowest frequency belonged to Oral-B (2.9±1.4). Kruskal Wallis test revealed that this difference was not statistically significant ($p<0.1$) and no significant differences were seen among other mouthwashes. Changes in the sense of taste were not significant ($p<0.15$).

Discussion

After about 40 years of use, CHX is still the gold standard for chemical plaque control [3]. The efficacy of CHX is due to its high substantivity in the oral environment and high antimicrobial activity [4]. The effective dosage of CHX is 20mg twice a day [16]. In Europe, 0.2% CHX has been introduced to the market and approved as the standard concentration. Lower concentration of CHX (0.12%) has also been evaluated in several studies and its efficacy has been confiremd as well [17, 18].

In the present study, epifluorescence microscope and SYTO 9/propidium iodide staining solution were used. Only limited studies have used this technique [12, 13, 19, 20].

Garcia et al. evaluated the efficacy of different concentrations of CHX on microbial flora and stated that the concentration and method of application of CHX can affect its antibacterial efficacy. In their study at 7h, the viable bacterial count was similar to the baseline value except for the 0.2% CHX group. Despite not eliminating the effect of confounding (external) factors namely eating, drinking and tooth brushing before sampling, substantivity of 0.2% CHX remained by up to 7h [19]. However, Tomas et al. showed that presence of confounding external factors (eating, drinking and chewing) decreased the antibacterial effect of 0.2% CHX at 3-7h and reported that this result questions the conventional prescription (twice daily) of CHX [13]. Our study also showed that the substantivity

of 0.2% CHX was higher than that of the two 0.12% CHX mouthwashes.

One of the most common side effects of CHX is change in the sense of taste and thus several manufacturers produced lower concentrations of CHX. Keijsor et al, [17] and van Strydonek et al. [18] believe that 0.12% concentration of CHX can have an efficacy similar to that of 0.2% CHX if increasingthe used volume from 10 to 15ml.

Harper et al. considered a washout period of 2.5 days for comparison of the efficacy of 0.2% and 0.12% CHX and found no significant difference in plaque index between these two concentrations [21]. In two other studies with 3-day [17] and 7-day [18] washout periods, no significant difference was noted either. The only differences between our study and that of Harper et al. was in the duration of washout period and the used amounts of the two concentrations of CHX as 15ml of the 0.12% and 10ml of the 0.2% CHX were used and no significant difference was found between the two;this result questions the use of volumes over 10ml. Qurilynen et al, also discussed this issue and found no significant difference in this respect [22].

Pizzo et al. [23] used 10ml of each of the two concentrations of CHX in a crossover study with a 10-day washout period and detected no significant difference in the incidence of dental plaque. In our study, no significant difference was found by using 10ml of both concentrations.

Studies on the CPC-containing mouthwashes have mainly compared them with a negative control group of saline solution [24, 25].

The results of our study are in concord with those of studies on CPC. The results of previous studies have shown that the activity of CPC-containing products ranks somewhere between the positive and negative controls similar to the finding of the present study. Certain similarities exist between the chemical and antimicrobial properties of CPC compounds and CHX. These similarities include positive charge of both and consequently high affinity to the oral environment. Gjermo et al. [26] stated that CPC had an effect similar or even greater than that of CHX on salivary bacteria. Thus, the difference in their anti-plaque activity cannot be justified merely by this characteristic. Bonesvoil and Gjermo evaluated the difference in clinical efficacy in a comprehensive study. They stated that although the release of CPC immediately after washing was significantly slower than CHX, its substantivity in saliva was significantly less than CHX. Thus, some factors must be present in the saliva that retard the clearance of CHX compared to CPC and are responsible for its higher substantivity [27].

Pan et al. demonstrated significant differences in the antibacterial properties of mouthwashes and reported that mouth rinses containing essential oils and CHX had higher anti-plaque activity compared to those containing CPC and combinations of CPC and CHX [28]. These results were also confirmed in our study.

Another substance becoming increasingly popular as a mouth rinse is Salvadora Persica or toothbrush tree extract. Its use by Middle Easterners goes back to 1400 years ago [6]. In a study by Seyedein and Shafieicomparing CHX and Persica it was demonstrated that both mouth rinses were effective for treatment of gingivitis after 6 weeks of usage and CHX was 2.5 times more effective for plaque removal than Persica [11]. In our study, CHX and Persica showed similar efficacy in the laboratory and clinical settings. It should be noted that in our study, the anti-plaque property of Persica was similar to that of CPC. Khoursand Salehi and Salehi Fard also discussed that both mouthwashes caused a significant reduction in pocket depth and papillary bleeding index but none had any effect on bone loss index [29]. In another study, plaque index after one week of using 0.2% CHX mouthwash as the only oral hygiene measure significantly decreased compared to the Persica group [30]. In this study, a significant reduction in plaque was noted as the result of using both concentrations of CHX compared to the Persica group. The difference of Persica and CPC with the two concentrations of CHX was mainly due to the substantivity of these two compounds and in case of requiring an efficacy similar to that of CHX, number of their daily applications should be increased (compared to CHX).

Patient cooperation required for eliminating the external confounding factors (eating, drinking and smoking) from an hour before the sampling to the end of sampling process was the main limitation of this study and we had no choice other than to trust patients. Moreover, difficult study conditions led to

loss of some cases to follow up and we had to replace them.

Another study is recommended to re-test our results in the clinical setting and evaluate the effect of external confounding factors on the substantivity of mouth rinses. Also, the estimated value of epifluorescence can be comparedwith microbial culture.

Conclusion

No significant difference was detected in antiplaque effects of 0.12% and 0.2% concentrations of CHX in the laboratory and clinical settings. CPC and Persica mouthwashes had similar antiplaque activity which was less than that of two different concentrations of CHX.

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