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A Comparison of antimicrobial effect of the mouthwash containing chlorhexidine, Cetylpyridinium chloride, and zinc lactate (Halita) and chlorhexidine against Pseudomonas aeruginosa and staphylococcus aureus: in vitro study

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Abstract: Staphylococcus aureous and pseudomonas aeroginosa have been dedicated the attentions since its potential to create oral diseases. Therefore the control of these microorganisms can play a great role in oral health. To gain this goal there are some medical methods such as utilizing a mouthwash like chlorhexidine. Since the chlorhexidine has some side effects like changing the taste, there is a new formula of cetyl predinium (Halita) which can be an alternative to chlorhexidine. The aim of this study was to compare the antibacterial effect of chlorhexidine and Halita on reduction of Staphylococcus aureous and pseudomonas aeroginosa. In this in vitro study there were 3 groups of chlorhexidine 0.2%, chlorhexidine 0.02% and halite as for positive control, negative control and the case respectively. Each mouthwash was pure up into 5 tubes in 90 ml and then 10 ml of bacterial species (0.5 Mc Farland) were mixed in with a suab and after that the specimens were picked up after 5, 10, 15, 30 and 60 seconds after mixing the bacterial species. The specimens were then cultured in plates in order to count the bacterial colonies. The data were analyzed by SPSS 17. The data revealed that the chlorhexidine 0.2% has a significantly great effect on staphylococcus and Halita showed the higher effect on pseudomonas aeroginosa. Nevertheless the overall results revealed that the halite and chlorhexidine 0.2% had a statistically significant difference in reduction of the bacterial counts. The study showed that the Halita can be an alternative mouth rinse to chlorhexidine 0.2% because of its less side effects.
Keywords: Staphylococcus aureous, Pseudomonas aeroginosa, Chlorhexidine, Halita.

INTRODUCTION

In addition to the bacteria lodging inside it, the oral cavity contains different pathogens including Pseudomonas aeruginosa and staphylococcus aureus, which are of medical importance especially in people with poor mouth hygiene, periodontal disease, and people with suppressed immune system [1]. Numerous studies have shown the relationship between Pseudomonas aeruginosa with chronic periodontal disease [2]. This organism might proliferate in the body and cause systemic infection such as bacterial pneumonia [3]. In patients suffering from cystic fibrosis, Pseudomonas aeruginosa causes pneumonia, urinary tract infection, bacteremia, and mortality due to chronic pulmonary infection and respiratory damage [4]. This strain has a high virulence and is able to produce extrahepatic enzyme and toxin to attach and form a biofilm onto tissue and mouth surfaces [5], and is resistant to the majority of antibiotics [6]. This pathogen has been found in refractory periodontitis [7]. Staphylococcus strains have been frequently isolated from the oral flora of children and adults [8, 9] whether in healthy individuals or in people suffering from disease such as rheumatoid arthritis and blood malignancies [10, 11]. Some infections originate from oral region developed by staphylococcus aureus. These infections include angular cheilitis (12), cardiac endocarditis [13], mandibular osteomyelitis [14] and mucositis parotid inflammation [15] in elderly individuals, and denture-dependent stomatitis [16]. The greater concern is observation of colonialization of staphylococcus aureus resistant to methicillin (MRSA) in oropharynx which is resistant to treatment and can be eliminated with difficulty [17]. Therefore, it seems that oral cavity is a less known reservoir for staphylococcus and Pseudomonas aeruginosa that cause topical or systemic infection under opportunistic conditions and also have the potential to penetrate from the oral cavity to other regions of the body. Mechanical washing for controlling oral biofilm and prevention from periodontal disease are recommended. However, the mechanical methods such as toothbrush and dental floss are not sufficient for prevention from development of dental biofilm and progression of oral diseases [18, 19]. One of the methods for improving mouth hygiene is the application of mouthwashes containing antimicrobial compounds such as chlorhexidine, ticlosa, and Cetylpyridinium chloride [20]. Chlorhexidine gluconate (CHG) mouthwash is considered as the golden standard thanks to its anti-plaque and anti-inflammatory properties for gum, though it can cause development of brown color in the mucosa and teeth, altered sense of taste, increased production of plaque, and development of mucous ulcers [21].

Cetylpyridinium chloride (CPC) mouthwash is a tetra-ammonium compound whose application has a grown throughout the recent decade. Laboratory studies have shown antibacterial effect of CPC on oral organisms and Helicobacter Pylori and the yeasts present in dental plaques 22]. In addition, clinical studies have indicated the influence of this mouthwash on development of plaque and gingivitis [23-25]. This compound is able to bond with hydroxy apatite, saliva, extracellular matrix, and diffuse into microbial biofilm [26, 27]. In order to improve the effects of their mouthwash, a combinational formulation containing CPC, CHX, and zinc lactate has been developed. This formulation has been evaluated in laboratory environment and has proven to enjoy antimicrobial properties. Furthermore, clinical studies have shown the ability of this mouthwash in preventing plaque formation, gingivitis, and bad breath [28, 29]. The commercial name of this product is Halita, which considering the low concentration of chlorhexidine in it, it not only enjoys antibacterial advantages, but also does not possess the disadvantages of chlorhexidine such as bad taste and altered dental color [30]. Since the studies conducted so far considering the effect of Halita on microbes involved in halitosis and there is no study on other microbial strains such as Pseudomonas aeruginosa and staphylococcus aureus, our aim in this study is to investigate the effect of mouthwash containing CPC and CHX on the levels of these two bacterial strains in laboratory environment, where chlorhexidine 0.2 mouthwash is used as the basis of comparison.

MATERIALS AND METHODS

In this study, probiotic capsules containing standard microbial strains of staphylococcus aureus and Pseudomonas aeruginosa were used. Further, the procedure was followed according to a similar study [31], in which considering the existence of different times and using the standard and similar conducted study, five times were selected for investigating the effect of mouthwashes, which are elaborated further.

The studied groups included: Group 1 (the control group), chlorhexidine 0.2% mouthwash group, Group 2 (the experimental group): the chlorhexidine 0.02% mouthwash group, Group 3 (the experimental group): Halita mouthwash group (Made by Rojin Co.).

In order to prepare chlorhexidine 0.02%, chlorhexidine 0.2% available in the market was mixed with 1-to-10 volume ratio with distilled water. This was done to experiment the effect of dilution of the mouthwash on its antibacterial effect.

Considering the two bacterial strains, the total number of tubes was as follows:

Five tubes containing chlorhexidine 0.2% for staphylococcus aureus, 5 tubes containing chlorhexidine 0.02% for staphylococcus aureus, 5 tubes containing Halita for staphylococcus aureus, 5 tubes containing chlorhexidine 0.2% for Pseudomonas aeruginosa, 5 tubes containing chlorhexidine 0.02% for Pseudomonas aeruginosa, 5 tubes containing Halita 0.2% for Pseudomonas aeruginosa

For investigations of time, a sample was taken from the first tube five seconds after dissolution of the bacterial with a swap. Considering the second tube, 10 s after dissolution, sampling was done. The next samples were taken from the 3rd to 5th tubes after 15, 30, and 60 s, respectively after dissolution of the bacteria. For the samples containing staphylococcus aureus, the taken sample was immediately cultured in solidified Agar culture medium by a microbiologist through four-quadrant streaking method using a sterile swap. For the samples containing Pseudomonas aeruginosa, the taken sample was immediately cultured in Trypton Soy Agar culture medium by a microbiologist through four-quadrant streaking method using a sterile swap. Next, the plates containing the culture medium were immediately transferred to an incubator 37°C and kept for 24 h.

After the growth of bacteria, colony counting was carried out through the following method:

STATISTICAL METHODS

The data obtained from the study were analyzed using descriptive statistics method (frequency-percentage and mean ± standard deviation) using Kruskal-Wallis test by SPSS 17. This is because distribution of the data was abnormal. Furthermore, at the end, Whitney test was employed for comparing the effect of the two types of mouthwashes. In this study, $p<0.01$ has been considered as statistically significant.

FINDINGS

The results of the effect of chlorhexidine 0.2% on the level of staphylococcus aureus and Pseudomonas aeruginosa are presented in Table 1. Kruskal-Wallis test indicates a significant difference in the frequency of staphylococcus aureus and Pseudomonas aeruginosa during various times of investigation.

Table-1: Comparison of the effect of chlorhexidine 0.2% mouthwashes on the level of staphylococcusaureus and Pseudomonas aeruginosa at various times

Pseudomonas aeruginosa	**Staphylococcus aureus**	**Time**
188.2 ±37.2	6.8±3.4	**5 sec**
162.47 ± 51.9	0	**10 sec**
155.8± 28.6	0	**15 sec**
70.61 ± 17.9	0	**30 sec**
2.9± 1.3	0	**60 sec**
P<0.01	P<0.01	P value*

P value*obtained from Kruskal-Wallis test

The results of the effect of chlorhexidine 0.02% on the level of staphylococcus aureus and Pseudomonas aeruginosa are presented in Table 2. Kruskal-Wallis test indicates a significant difference in the frequency of staphylococcus aureus and Pseudomonas aeruginosa during various times of investigation.

Table-2: Comparison of the effect of chlorhexidine 0.02% mouthwashes on the level of staphylococcusaureus and Pseudomonas aeruginosa at various times

Pseudomonas aeruginosa	**Staphylococcus aureus**	**Time**
>100000	>100000	**5 sec**
>100000	>100000	**10 sec**
>100000	20000± 8467	**15 sec**
>100000	1645± 638	**30 sec**
80000± 12467	1509± 422	**60 sec**
-	P<0.01	P value*

P value*obtained from Kruskal-Wallis test

The results of the effect of Halita on the level of staphylococcus aureus and Pseudomonas aeruginosa are presented in Table 3. Kruskal-Wallis test indicates a significant difference in the frequency of staphylococcus aureus and Pseudomonas aeruginosa during various times of investigation.

Table-3: Comparison of the effect of Halita mouthwash on the level of staphylococcusaureus and Pseudomonas

Pseudomonas aeruginosa	**Staphylococcus aureus**	**Time**
3.7 ±1.2	104.3± 48.5	**5 sec**
11.6 ± 4.7	56.8± 23.4	**10 sec**
8.5± 2.9	55.9± 17.3	**15 sec**
7.4± 3.1	20± 9.4	**30 sec**
0	1.5±.46	**60 sec**
P<0.01	P<0.01	P value*

P value*obtained from Kruskal-Wallis test

The results of Tables 4 and 5 indicate that:

The degree of the effect of the three types of mouthwashes on staphylococcus aureus during 60 s had a significant difference with each other (P=0.032). As the Mann-Whitney test (Table 4.5) indicates, considering staphylococcus aureus, chlorhexidine 0.2% mouthwash had significantly the greatest disinfection properties, whereas chlorhexidine 0.02% had significantly the lowest level of disinfection properties.

The degree of the effect of the three types of mouthwashes on Pseudomonas aeruginosa during 60 s had a significant difference with each other (P=0.008). As the Mann-Whitney test (Table 4.5) indicates, considering Pseudomonas aeruginosa, Halita mouthwash had significantly the greatest disinfection properties, whereas chlorhexidine 0.02% had significantly the lowest level of disinfection properties.

Table-4: Comparison of the effect of three mouthwashes on the level of staphylococcusaureus and Pseudomonas aeruginosa during 60 s

Pseudomonas aeruginosa	**Staphylococcus aureus**	**Time**
1.2 ±2.7	115 ± 77.05	**chlorhexidine%0/2**
7700 ±10652.23	80000±8944.27	**chlorhexidine %0/02**
44.2± 37.53	5.8 ± 4.32	**Halita**
P=.008	P=0.032	P value*

P value*obtained from Kruskal-Wallis test

Table-5: The Mann-Whitney test for paired comparison of mouthwashes considering the effect on staphylococcus aureus and Pseudomonas aeruginosa

P value	Mean difference	Mouthwash	Mouthwash	
.022	7698.80	**chlorhexidine%0/2**	**chlorhexidine %0/02**	**Staphylococcus aureus**
0.036	7655.80	**Halita**	**chlorhexidine %0/02**	
0.016	-43.00	**Halita**	**chlorhexidine %0/2**	
0.000	79885.00	**chlorhexidine%0/2**	**chlorhexidine %0/02**	**Pseudomonas aeruginosa**
0.000	79994.20	**Halita**	**chlorhexidine %0/02**	
0.026	109.20	**Halita**	**chlorhexidine %0/2**	

P value*Obtained from Mann-Whitney test

DISCUSSION

This study indicated that Halita mouthwash had a reasonable effect during 60 s on staphylococcus aureus and Pseudomonas aeruginosa. Over the past few years, studies have shown that existence of non-oral strains in oral cavity is not transient or the result of contamination during the sampling, and thus oral cavity is the reservoir for pathogens that are of medical importance. Staphylococcus aureus is a gram-positive bacteria frequently isolated from sub- and epi-gingival plaque [32]. Methicillin-resistant staphylococcus becomes colonized across denture mucous surfaces and is eliminated very difficult through conventional therapies [16]. The data related to periodontal infections and colonialization of Pseudomonas aeruginosa have shown that prevalence of such strains in the oral cavity causes periodontitis to be observed with a greater intensity in these individuals [33]. This strain is the cause of failure in treatment for patients suffering from periodontitis resistant to mechanical and antimicrobial treatments [7]. It is believed that the high degree of resistance to broad-spectrum and numerous antibiotics along with the biofilm abilities might result in persistence of this strain in periodontal packet after treatment [5]. Therefore, adequate care is required in these patients in order to reduce the risk of periodontal disease and progression of systemic diseases through these strains. Further, the applied antibiotic should also cover this organism.

Conventional antibiotic therapies for oral and dental diseases are usually prescribed in oral and mouthwash forms. Typically, the most effective method is mouthwash, since it is easier to be used by patients in terms of time. Therefore, focus on mouthwash for antibiotic therapy appears to be important. Studies have shown that application of chlorhexidine 0.2% leads to elimination of methicillin-resistant staphylococcus [34]. Albequerque *et al*. have revealed that chlorhexidine 12% mouthwash is able to curb staphylococcus aureus in in-vitro environment [35]. In a study by Herrea *et al*., the effect of four mouthwashes containing chlorhexidine 12% with different formula in laboratory environment on 20 microbial strains was evaluated. It was found that adding CPC to chlorhexidine enhances its antimicrobial activity [36].

In this study, Pseudomonas aeruginosa and staphylococcus bacteria were examined. This was due to investigation of the effect of this mouthwash, since the effect of Halita on common bacteria such as Streptococcus mutans has already been investigated and its positive effect has been proven [37]. Therefore, if as with chlorhexidine, it was able to present a good effect on Pseudomonas aeruginosa and staphylococcus aureus strains, considering its relative advantages versus chlorhexidine 0.2%, it would have been introduced as a suitable and superior mouthwash in oral antibiotic treatments. According to the results obtained and presented in Table 4.1, chlorhexidine has gained acceptable results. As in other studies such as Abbaszadegan *et al*. such a test has been carried out on chlorhexidine 0.02%, similar results considering the effect of chlorhexidine 0.2% mouthwash on Pseudomonas aeruginosa and staphylococcus aureus have been obtained. Therefore it can be concluded that this study enjoys the required standard considering judgment on the results [38].

On the other hand, one of the mouthwashes has been chlorhexidine 0.02%. The reason of examining this mouthwash was comparing it with chlorhexidine 0.2%. This is because this concentration of chlorhexidine is observed in some mouthwashes and it is examined whether this concentration of chlorhexidine can be reduced for removing or mitigating its adverse effects or not. Similar to this work has been done by Witt *et al*. They found that reducing the concentration of chlorhexidine not only is not able to decrease its adverse effects, but also completely eliminates its effectiveness [31]. The results of our study also confirmed this matter at least in terms of effect on Pseudomonas aeruginosa and staphylococcus aureus bacteria. Therefore, it is recommended not to decrease the concentration of chlorhexidine.

Furthermore, in this study examination of the mouthwashes was carried out across five different times. Similar to this work has been performed by Witt *et al*, due to application of the obtained results as the suitable time to be labeled on the final commercial product. According to the results obtained from our study, chlorhexidine 0.2% mouthwash has been well effective on staphylococcus after 10 s, but on Pseudomonas aeruginosa, it required at least 30 s in order for its positive effect to emerge. Similar to these results can be observed in the study by Witt J *et al*. However, considering chlorhexidine 0.02% and Halita this duration was longer, such that in Halita at least 30 and 60 s were required for removing staphylococcus and Pseudomonas aeruginosa, respectively, in order for the mouthwash to have significant effects on the bacteria. Nevertheless, these results regarding Halita were in contrast to those obtained by Stooky *et al*. [39], the reason of this difference might lie in the fact that first their study was a clinical trial and second the type of Halita mouthwash has been different. On the other hand, according to the obtained results, the ideal time for the three mouthwashes has been 30, 60, and 60 s for chlorhexidine 0.2%, Halita, and chlorhexidine 0.02%, respectively, though 60 s of applying Halita removes virtually all colonies, but chlorhexidine 0.02% merely decreases this value when compared with other times. Therefore, it can be concluded that for chlorhexidine 0.02% considering the fact that the reduction in the staphylococcus aureus and Pseudomonas aeruginosa bacteria took place within 15 and 60 s, respectively, but considering the total number of bacteria grown in the plates in comparison with chlorhexidine 0.2% and Halita, this extent has been far larger and it can be stated that it has had virtually no effect on the studied bacteria. This degree might have decreased over time, but over time the popularity of a mouthwash declines in return and it is not logical medically nor commercially. However, further studies are required to compare this mouthwash other mouthwashes during over 60 s so that the accurate efficiency of this mouthwash is proven.

In order to clarify the effect of the three mouthwashes, through Mann-Whitney test, these mouthwashes underwent paired comparison. According to that, considering staphylococcus aureus and Pseudomonas aeruginosa, chlorhexidine 0.02% and Halita, respectively had the greatest effect. However, chlorhexidine 0.02% showed the weakest effect in both cases. Based on the two tests, it is possible to evaluate the results obtained from chlorhexidine 0.2% and Halita as acceptable versus chlorhexidine 0.02%.

In conclusion, it can be deduced that Halita and chlorhexidine 0.2% have achieved successful results experimentally. In spite of this finding, it can be stated that Halita mouthwash can be benefited from as a suitable mouthwash for oral antibacterial therapies and especially gum diseases thanks to being free of drawbacks of chlorhexidine such as altered sense of taste.

At the end, it should be said that this research has shown the effect of Halita mouthwash, but this effect has been gained in laboratory and test tubes. Naturally, different intra-oral factors and the systemic conditions of patients can also influence the obtained results. Therefore, the need for further studies especially clinical studies considering the proof of this effect is felt.

CONCLUSION

According to the obtained results, it can be concluded that Halita and chlorhexidine 0.2% mouthwashes act similarly in terms of removing Pseudomonas aeruginosa and staphylococcus aureus bacteria. With regard to the low adverse effects of Halita, it can be introduced as a suitable mouthwash in antibacterial treatments.

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