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Original Article

The Antibacterial Analysis of Alcohol-Free and Alcohol-Based Chlorhexidine Mouthwashes Against Oral Bacteria

Hojatollah Yousefimanesh[1*], Maryam Robati[2], Atefeh Piri[3], Azardokht Khosravi Boroujeni[4], Mehrandokht Sirous[5]

[1]Department of Periodontology, School of Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Oral Medicine, School of Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Department of Periodontology, School of Dentistry, Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[4]Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[5]Department of Microbiology, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran

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***Corresponding author:**
Hojatollah Yousefimanesh, Assistant Professor, Department of Periodontology, School of Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Tel: +989304874007,
Email: hojjatyoosefi@yahoo.com

Abstract

Background: Chlorhexidine (CHX) is the gold standard chemical agent against oral pathogenic bacteria and is widely used for plaque/gingivitis control. The aim of the present study was to compare the effect of alcohol-based and alcohol-free CHX mouthwashes on oral microorganisms.

Methods: In the present *in vitro* study, the standard strains of four microorganisms present in the oral cavity were prepared, including *Streptococcus mutans, Streptococcus sanguinis, Streptococcus salivarius,* and *Lactobacillus casei*. The serial dilutions of CHX antimicrobial agents were obtained, and the level of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined using the broth dilution method. Finally, data were analyzed using the Kruskal-Wallis test, the Mann-Whitney U test, and SPSS-16.

Results: The MIC values of 0.12% and 0.2% alcohol-free CHX and 0.2% alcohol-based CHX for *S. mutans* were 1.17, 0.48, and 0.24 µg/mL, respectively. The MBC values of 0.12% and 0.2% alcohol-free CHX and 0.2% alcohol-based CHX for *S. mutans* were 18.78, 7.81, and 7.81 µg/mL, respectively. The MIC and MBC values of the tested CHX mouthwashes for *S. mutans* were significant ($P \leq 0.05$).

Conclusions: Overall, the 0.2% alcohol-based CHX mouthwash had the highest antibacterial activity against gram-positive bacteria.

Keywords: Chlorhexidine, Mouthwashes, Minimum inhibitory concentration, Minimum bactericidal concentration, Alcohol

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Background

The human oral cavity with a specialized oral environment is the habitat of a wide range of microorganisms, including bacteria. The metabolic activity of some species of bacteria can promote the growth of other species of bacteria (1). The community of microorganisms and biofilms in close collocation on host surfaces can create a microbial plaque, which can initiate the destruction of periodontium structures (2). Various microorganisms have been recognized in the oral cavity, including *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, and *Lactobacillus casei*. They are gram-positive facultative anaerobic bacterium, gram-positive facultative anaerobic coccus species of bacteria and a member of the viridans *Streptococcus* group, gram-positive, facultative anaerobic bacterium, and gram-positive, non-motile, non-sporulating and catalase-negative bacterium, respectively (1,3).

Mechanical and chemical oral hygiene techniques have been realized for the control and removal of plaque biofilms and prevention of dental plaques built up on the tooth surface and along the gingival margin. Mechanical plaque control is the common and primary method for the removal of dental plaques and prevention of supragingival biofilm development. Toothbrushes, interdental brushes, dental floss, wood stick, and rubber interdental bristles are the most widespread standard tools in mechanical plaque control methods (3). Despite the importance of the mechanical plaque control method, gingivitis is highly prevalent due to improper plaque control and physical disability. Therefore, it is recommended to use the chemical plaque control method to maintain hygiene and increase the efficiency of mechanical methods. The most common chemical agents for plaque control are toothpaste and mouthwashes with anti-inflammatory and anti-plaque properties (4,5).

Mouthwashes with antiseptic, inhibiting, and preventive plaque properties can interfere with the initial bacterial adhesion to the tooth surface and reduce various stages of plaque formation. Moreover, the anti-plaque, anti-inflammatory, and anti-microbial properties of mouthwashes have been confirmed in various clinical studies (6,7). The active ingredients of most available mouthwashes are cetylpyridinium chloride, chlorhexidine (CHX), essential oils, fluoride, and peroxide (8,9). CHX mouthwash has been recognized as the promising chemical antibacterial agent that affects Gram-positive and -negative bacteria (10-12). Despite numerous advantages of the CHX mouthwash, CHX has some potential side effects such as tooth staining, taste alteration, burning mouth, and supragingival calculus formation (13,14).

Some mouthwashes contain a specific concentration of alcohol that acts as an antiseptic agent. Some studies suggested that the alcohol content contributes to the stability, maintenance, and effectiveness of CHX. However, other studies recommended alcohol-free products and concluded that the risk of the oral cavity, pharyngeal, and laryngeal cancers is associated with alcohol-based mouthwashes (15,16). Modern mouthwashes have shown to be effective in removing plaques, reducing gingivitis, and freshening breath. Therefore, the choice of alcohol-containing mouthwashes versus alcohol-free mouthwashes is necessary for maintaining proper oral health. In this regard, the current *in vitro* study aimed to examine the effect of alcohol-based and alcohol-free CHX mouthwashes on oral microorganisms.

Methods

In the present *in vitro* study, the standard strains of four microorganisms present in the oral cavity were provided, including *S. mutans*, *S. sanguinis*, *S. salivarius*, and *L. casei*. The study was conducted in accordance with the ethical principles and guidelines of the World Medical Association Declaration of Helsinki and confirmed by the Ethics Committee of Ahvaz Jundishapur of Medical Sciences.

A total of three types of mouthwashes were selected for this study, including 0.2% alcohol-free CHX mouthwash (Shahre Daru, Tehran, Iran), 0.2% CHX mouthwash with alcohol (Najo Daru, Tehran, Iran), and 0.12% alcohol-free CHX (Epimax, Emad Company, Iran).

Microbial Culture Suspension Preparation

A standard operating procedure was performed to prepare the microbial culture suspension (17). The bacterial strains of the oral cavity were prepared from the Persian type culture collection (PTCC), including standard strains of *S. mutans* (PTCC1683), *S. sanguinis* (PTCC1449), *S. salivarius* (PTCC1448), and *L. casei* (PTCC1608).

Each bacterial strain was isolated and cultured in the tryptic soy broth (TSB) medium. A volume of 1 mL of sterile TSB (Merck, Germany) was added to 20 sterile tubes. The serial dilution method was used for the minimum inhibitory concentration (MIC) of the mouthwashes. Afterward, 1 mL of each mouthwash with specified dilutions was added to the sterile tubes.

The bacterial suspension was adjusted to 0.5 McFarland standard (*1.5 × 10⁸ colony-forming units [CFU*/mL]) diluted using 0.002 physiological serum. Bacterial suspensions in a dilution of 10^6 CFU/mL were obtained following diluting. Then, 1 mL of the diluted suspension was added to the test tubes containing the TSB medium and mouthwash and mixed for one minute. The tubes were labeled using the sample reference number. The test tubes were incubated at 37°C for 48 hours. To determine the growing microorganisms in the culture, the color and turbidity of test tubes were analyzed by visual examinations. Next, the turbidity measurements were performed at the same condition (medium, temperature, and shaking speed). The test tube with the lowest concentration of the mouthwash and clear supernatant was considered as the MIC value, and the MIC of the studied mouthwashes was measured and recorded accordingly. The test tubes with a clear supernatant (without turbidity) were transferred to a solid blood agar culture medium (Merck, Germany) for the measurement of the minimum bactericidal concentration (MBC) of mouthwashes. This process was performed three times for all studied bacterial strains.

Data Analysis

Data were analyzed by the Kruskal-Wallis test and the Mann-Whitney U test. The results were tabularized and analyzed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA, and a *P* value less than 0.05 (typically ≤ 0.05) was considered statistically significant.

Results

The antibacterial analysis of the studied mouthwashes showed that all three types of mouthwash had inhibitory effects against oral cavity microorganisms. The MIC and MBC values of the tested mouthwashes against bacterial strains are provided in Table 1. The alcohol-based CHX mouthwash (0.2%) exhibited high activity (the lowest MIC value) against the strain of *S. mutans* (MIC = 0.24 µg/mL) compared to other studied mouthwashes. The alcohol-free CHX mouthwash (0.12%) demonstrated lower activity (the highest MIC value) against *S. sanguine* (MIC = 2.34 µg/mL). The alcohol-free CHX (0. 2%) and alcohol-based CHX (0.2%) mouthwashes revealed the MBC against the strain of *S. sanguine* (MBC = 1.95 µg/mL, Table 1). Eventually, the alcohol-free CHX mouthwash (0.12%) represented the highest MBC value against the strains of *S. mutans* and *L. casei* (MBC = 18.75 µg/mL, Table 1).

The pairwise comparison of mouthwashes is presented in Table 2. The pairwise comparison of mouthwashes indicated no significant difference between the MIC values of alcohol-free CHX (0.2%) and alcohol-based CHX (0.2%) mouthwashes against all strains of bacteria, except for *S. mutans* ($P \geq 0.05$). In addition, there was no significant difference between the MBC values of alcohol-free CHX (0.2%) and the alcohol-based CHX (0.2%) mouthwashes

Table 1. The MIC and MBC Values (µg/mL) of Alcohol-Free and Alcohol-Based CHX Mouthwashes Against Oral Bacterial Strains

	Bacteria	0.2% Alcohol-Based CHX	0.2% Alcohol- Free CHX	0.12%Alcohol- Free CHX
MIC (µg/mL)	*Streptococcus mutans*	0.24	0.48	1.17
	Streptococcus sanguinis	0.48	0.97	2.34
	Streptococcus salivarius	0.97	0.48	1.17
	Lactobacillus casei	0.48	0.97	1.17
MBC (µg/mL)	*Streptococcus mutans*	7.81	7.81	18.75
	Streptococcus sanguinis	1.95	1.95	4.68
	Streptococcus salivarius	7.81	7.81	9.37
	Lactobacillus casei	15.62	15.62	18.75

Abbreviations: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; CHX, chlorhexidine.

Table 2. The Pairwise Comparison of Mouthwashes Against Oral Bacterial Strains

	Pairwise Comparison of Mouthwashes	*Lactobacillus casei*	*Streptococcus salivarius*	*Streptococcus sanguinis*	*Streptococcus mutans*
MIC (*P* value)	0.2% alcohol-based and alcohol- free CHX	0.571*	0.571*	0.571*	0.012
	0.12% alcohol-free CHX and 0.2% alcohol-based CHX	0.041	0.024	0.018	0.002
	0.12% alcohol-free CHX and 0.2% alcohol-free CHX	0.024	0.041	0.024	0.041
MBC (*P* value)	0.2% alcohol-based and alcohol-free CHX	0.8*	0.67*	0.7*	0.8*
	0.12% alcohol-free CHX and 0.2% alcohol-based CHX	0.031	0.041	0.036	0.02
	0.12% alcohol-free CHX and 0.2% alcohol-free CHX	0.031	0.041	0.036	0.02

Abbreviations: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; CHX, chlorhexidine.
*Not significant ($P > 0.05$).

against all strains of bacteria ($P \geq 0.05$). The alcohol-based CHX mouthwash (0.2%) showed the lowest MIC, and therefore, better antibacterial activity against *S. mutans* compared to the alcohol-free CHX mouthwash (0.2%). On the other hand, the alcohol-free CHX mouthwash (0.12%) demonstrated the highest MIC value, implying the lowest activity or no activity of the alcohol-free CHX mouthwash (0.12%) against biofilm strains compared to the other two studied types of mouthwash.

Based on the comparative analysis of oral bacteria, *L. casei* and *S. mutans* had the highest and lowest resistance to alcohol-based and alcohol-free 0.2% CHX mouthwashes, respectively. Moreover, *S. sanguis* and *L. casei* revealed the highest resistance, while *S. mutans* displayed the lowest resistance to the 0.12% alcohol-free CHX mouthwash.

Discussion

The present study focused on examining the antimicrobial activity of three common CHX mouthwashes with and without alcohol against oral gram-positive bacteria.

All three studied mouthwashes (0.2% alcohol-free CHX, 0.2% alcohol-based CHX, and 0.12% alcohol-free CHX) showed inhibitory activity against the strains of oral bacteria. There was a significant difference between the antibacterial activities of the mouthwashes. The 0.2% alcohol-based CHX mouthwash represented promising antibacterial activity, which could be due to the high concentration of CHX and the inclusion of alcohol as an antiseptic agent.

Streptococcus and *L. casei* have the potential to attach to the surface of teeth and oral mucosa. These bacteria can multiply and produce a sticky matrix, which shifts to a complex mixed population biofilm (18). Bacterial growth and colonization of bacterial cells around the tooth surface contribute to the formation of dental bacterial plaques. In the process of plaque formation, filaments and spirochetes cannot directly attach to the surface of teeth, therefore, they attach and preponderate in the exterior surface of plaque mass, resulting in the formation of a fully mature oral biofilm (19). Bacterial plaque is the leading cause of periodontal disease and tooth decay. The most commonly used plaque biofilm removal/control methods are mechanical and chemical approaches. Despite the advantages of the mechanical plaque control method, some limitations are attributed to its bactericidal activity, thus the chemical method has been introduced as an alternative/adjunctive antibacterial method. Mouthwashes are the most widely used antiseptic or antibacterial chemical agents that can be employed as an effective adjunct to the mechanical plaque control method (20).

The mechanism of action of mouthwashes includes the disruption of bacterial metabolism, inhibition of cell growth, and death of the cell. The bactericidal and/or bacteriostatic action of mouthwashes depends on their active components and their concentrations (21).

The CHX mouthwash is the most effective and widely applied as a gold standard antiseptic agent for plaque control (9,22). Moeintaghavi et al suggested that CHX possessed antibacterial activity at all concentration levels (23), which is consistent with the results of the present study. In another study, Amin et al compared the effect of garlic juice and CHX mouthwash on oral pathogens and concluded that the garlic extract and CHX mouthwash had antimicrobial activities against oral pathogens (24).

Various studies have examined the relationship between alcohol mouthwash and the activity of CHX solutions with different concentrations, but the results are still controversial. Najafi et al compared the efficacy of two concentrations of CHX mouthwashes (0.12% and 0.2%) on gingival indices and the level of dental staining and found that plaque index and gingival index significantly reduced by two concentrations of CHX mouthwashes (0.12% and 0.2%), and the two concentrations did not significantly differ from each other. However, 0.2% CHX was reported to be more efficient in the gingival bleeding index compared to 0.12% CHX (25). Likewise, Haydari et al suggested that the efficacy of CHX mouthwash containing 0.2% in preventing dental plaque was better in comparison with 0.12% solutions (26). Todkar et al concluded that the alcohol-free mouthwash was as effective as the alcohol-based mouthwash in plaque control and reduction of gingival inflammation, which is inconsistent with the results of the present study. This could be due to the difference in the type of the study and the effect of a clinical study on the bioactivity of mouthwashes (27). To confirm this claim, Leyes Borrajo et al showed that the dose and concentration of mouthwashes can influence the activity of mouthwashes in addition to their active ingredients (28). Therefore, the chemical composition of mouthwashes and the type of the study (*in vitro* or *in vivo*) can influence the results of the study. Similarly, in an *in vivo* study, Cousido et al reported that mouthwashes containing 0.2% and 0.12% CHX were released immediately after rinsing the mouth, but the substantivity of 0.2% CHX in the oral cavity was more than 0.12%, highlighting the effects of the concentration on the survival and duration of mouthwash in the mouth (29).

In a systematic review, Berchier et al evaluated the effects of 0.12% and 0.2% CHX mouthwashes and demonstrated a significant difference in the 0.2% CHX concentration on the plaque and periodontal indices (30). Ghasempour et al also confirmed the inhibitory activity of 0.2% CHX against *S. mutans* (31), which corroborates with the results of the current study. In the present study, *S. mutans* represented the most sensitivity to CHX compared to other tested bacteria. *S. mutans* is the main organism of the oral cavity that contributes to the formation of plaque biofilms and initiation of dental caries, and its inhibition will disrupt the formation of cariogenic dental plaques (32). Accordingly, CHX mouthwash, particularly alcohol-based CHX, is recommended for oral hygiene and the prevention of dental plaques (33,34).

Conclusions

The antibacterial analysis of 0.2% alcohol-free CHX mouthwash, 0.2% alcohol-based CHX mouthwash, and 0.12% alcohol-free CHX showed that all three types of mouthwash had inhibitory effects against oral cavity microorganisms. Finally, the 0.2% alcohol-based CHX mouthwash indicated the highest antibacterial activity against oral bacteria.

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Conflict of Interests

The authors declare no conflict of interests.

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