COMPARISON OF ANTIMICROBIAL EFFECTS OF MOUTHWASHES

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ABSTRACT

**BACKGROUND**:  This study investigated the in vitro and in vivo antibacterial effects of three mouthwashes on supragingival plaque microbiota. The three mouthwashes under study were 0.2% Chlorhexidine (CHX), Listerine®, and Persica (PM). Water was used as negative control. **MATERIALS AND METHODS**: Supragingival plaque samples were collected from 32 patients with gingivitis. Plaque samples were swabbed on agar plates and discs (previously immersed in the three mouthwashes) were placed on the agar. The zone of bacterial inhibition (ZOI) was measured after incubation for 24 hours. The same plaque samples were inoculated on agar and the colony forming units (CFU) were counted for the in vivo testing. The patients were then instructed to use the mouthwashes (cases) and water (controls) for two weeks, after which plaque samples were again collected, inoculated and the CFUs were counted. **RESULTS:** The results revealed that for the ZOI test 0.2% CHX inhibited the growth of bacteria to an average diameter of 18.38 mm,  while Listerine®, PM and water caused no inhibition of bacterial growth around the discs after 24 hours. The mean bacterial count after using 0.2% CHX for two weeks decreased by 23.13. This was followed by Listerine®, with a mean reduction of 19.75. PM resulted in 13.5 decrease in the mean bacterial count, while water reduced the bacterial count by only 1. **CONCLUSION**: Based on the results, 0.2% CHX inhibits bacterial growth considerably. All three mouthwashes can reduce total bacterial count after 2 weeks although with different mean bacterial count reduction.

**Introduction:**

 Antonie van Leeuwenhoek was the first to announce the presence of bacteria in the oral cavity in 1699 and described the presence of ‘living animalcules’ in dental plaque (1). The microbial etiology of periodontal disease has been a subject of interest to researchers for over a hundred years and has significantly evolved with recent advances in bacterial identification and characterization (2). According to the current concept regarding the etiology of periodontal disease, three factors determine the risk of active periodontal disease namely a susceptible host, presence of pathogenic species, and absence of so-called "beneficial bacteria" (3). Bacteria colonize the oral cavity within a few hours after birth. Colonization of the gingival crevice occurs initially by bacterial interactions with the tooth and later by interbacterial interactions leading to the formation of an organized, symbiotic community called biofilm. Current evidence indicates that gingivitis and periodontitis are polymicrobial infections caused by the biofilm-associated bacteria (2).

 Gram-positive cocci, especially *Streptococcus spp*. and *Actinomyces spp.* are the dominant flora of healthy gingival sulcus. While the microbial flora of a mature plaque consists of facultative anaerobic microorganisms, spirochaetes and motile rods. Strict anaerobic, Gram-negative and motile organisms significantly increase as the disease progresses (3). In order to prevent periodontal disease, elimination of dental plaque is necessary by mechanical and chemical methods. It has been widely known that use of antimicrobial oral rinses plays an important role in maintaining oral hygiene mainly by reducing the number of dental plaque microorganisms. Mouthwashes are very useful in reducing microbial plaque. Among the available mouthwashes, CHX has shown to be effective in reducing dental plaque and pathogenic microorganisms including *Streptococcus mutans* (4).

 The mechanism of action of CHX involves interactions with external cell components and the cytoplasmic membrane, causing a high rate of leakage of intracellular components, and interactions with cytoplasmic constituents. Damage to the outer cell layers alone is insufficient to induce cell death and CHX uptake is dependent upon both the pH and the concentration of the solution (5).

 Considering the side effects of CHX, search for new and alternative antimicrobial substances with less side effects continues. Another frequently used mouthwash, usually recommended as a part of home-care oral hygiene regimen, is Listerine®. Fornell et al. (1974)(6) stated that, significantly lower Plaque and Gingival Index values were scored with Listerine and lower amounts of plaque could be sampled in comparison to the controls. Kasuga  et al. (1997)(7) reported that rinsing Listerine® for 30 seconds resulted in a decrease of approximately 0.01 of the viable bacterial counts in the saliva. These bactericidal effects in the saliva and dental plaque indicated that Listerine® and Cool Mint Listerine antiseptic are useful as antiseptic mouth rinses. When comparing Listerine® with a CHX-based mouthwash (Peridex®), Balbuena et al. (1998)(8) demonstrated that both mouthwashes significantly reduced bacterial counts one hour after use by volunteers. At 4 hours after treatment, Peridex® oral rinse showed a further reduction in the bacterial colony count in the saliva whereas Listerine® antiseptic showed no difference compared with normal saline solution. In a recent investigation, Aneja et al. (2010)(9) found Listerine to be effective against *Streptococcus mutans and Staphylococcus aureus* at four different concentrations.

 Wood sticks are traditionally and widely used for cleaning the teeth in several countries in the Middle East and Africa. The plant most commonly used as cleaner is Salvadora persica, a small tree, growing wild with a very wide geographical distribution. It has been used for many centuries by different communities as an oral hygiene aid. The therapeutic effect of persica could be due to certain chemical constituents such as fluoride, silicones, essential alkaloids, tannins, resins and anthraquinones. It has been shown that using this herb or its extract could support periodontal health, and reduces the accumulation of microbial plaque as well as bleeding during brushing (10).

 The objective of this study was to compare the antimicrobial effects of 0.2% CHX, persica mouthwash (PM) and Listerine® on aerobic and facultative bacteria from supragingival plaque of patients with gingivitis.

**Materials and Methods**

Hypersensitivity testing of the subjects to the mouthwashes, collection of plaque samples, as well as oral hygiene instructions and follow-up were done at the University of the East Post Graduate School Clinic in March 2010. Sample processing and all other laboratory procedures were done at the Department of Clinical Microbiology Laboratory, Mary Chiles Hospital.

**Sources and samples:**

 The participants in this study were recruited from patients seeking periodontal treatment at the Graduate School of the University of the East, Department of Periodontics. They signed an informed consent form provided by the university. The samples were gathered from supragingival plaque of patients with gingivitis. The eligible subjects were selected based on the following clinical parameters:

* Erythema
* Bleeding on probing
* Age between 25-35 years old
* Moderate plaque index (40-70%)
* No bone loss

Exclusion from the study was based on the following criteria:

* Smoking
* Systemic disease
* Pregnancy and lactation
* Orthodontic or prosthodontic appliances
* Antibiotic therapy within the past three months
* Adverse reaction to the three mouthwashes

**Instrument for gathering data and validation:**

 Supragingival samples were collected with a sterile curette with the aid of a mouth mirror and cotton rolls. A sterile tube was used for transferring the samples to the laboratory.

**Procedure for gathering data:**

 For standardization, first, the patients were given oral prophylaxis. Oral B toothbrush and toothpaste were given to all patients and subjects were instructed to brush their teeth by the modified Bass technique three times in a day and use mouthwashes twice a day according to the manufacturer’s instructions. Plaque index was measured every 5 days. The samples were gathered before and two weeks after using the mouthwashes.

 The 32 patients were randomly divided into four groups (eight patients in each group) using CHX, PM, Listerine® and water. Each group of patients used the mouthwashes every 12 hours as follows:

CHX group: 30 seconds mouth rinsing with 30 ml of CHX mouthwash.

PM group: 20 seconds mouth rinsing with 15 drops of PM in 15 ml of water.

Listerine® group: 30 seconds mouth rinsing with 20 ml of Listerine mouthwash.

Control group: 30 seconds mouth rinsing with 30 ml of water.

**Sample Collection:**

 Supragingival plaque sample equal to 1mg was collected with a sterile curette and directly immersed in a sterile vial containing 1ml of phosphate buffered saline (PBS).

To disperse the bacterial cells, the solution was homogenized by an agitator for five minutes. Ten-fold serial dilutions were made in PBS with a repeat homogenization on the agitator for 60 s at the start and between successive dilutions. Dilutions of 1 x 10-5 were then used for culturing.

**Preparation of Mueller Hinton** **agar plates and inoculation of bacteria**

For bacterial colony counting, 0.1 ml of diluted samples were transferred into empty plates. Mueller Hinton agar was cooled to 50° C, and poured into each plate. After the agar had solidified, the plates were incubated for 24 hours and the colony forming units (CFU) were counted. The same method of sample collection and inoculation was repeated after two weeks when the subjects had completed a two-week regimen of mouthwash, or water use.

The zone of growth inhibition (ZOI) test was done in vitro, before the patients used the mouthwashes or water. The same procedures for sample collection and dilution were followed. Bacteria were streaked on the agar surface with a swab. The plates were then divided into four equal sections, labeled as C, L, P and W for CHX, Listerine®, PM, and water, respectively. Filter paper discs impregnated with each of the mouthwashes and water were then placed at the center of each section and the discs were pressed lightly on to the agar.

 Then the plates were incubated in the inverted position at 370 C for 24 hours. After 24 hours, ZOI around the disc was measured. The measurement was between disc edge and growth**.**

**Statistics for analyzing data**

Data collected were studied by coding the variables in Excel format and using the pHstate2 software to obtain the descriptive statistic analysis. The difference between the four groups was determined using the analysis of variance (ANOVA).P-values less than 0.05 were considered statistically significant.

**Results**

 Table 1 summarizes the bacterial counts before and after two weeks use of 0.2% CHX, Listerine**®** and Persica mouthwash in patients with gingivitis.

|  |  |  |
| --- | --- | --- |
|  |  Before |  After |
|  Mean± SDCHX Min/Max Count |  26.88±7.59 21/43 8 |  3.75±2.25 1/8 8 |
|  Mean± SDListerine Min/Max Count |  27.5±6.25 19/36 8 |  7.75±2.66 4/12 8 |
|  Mean± SDPM Min/Max Count |  22.13±7.88 12/32 8 |  8.63±3.11 4/13 8 |

 The summary statistics on the bacterial counts before and two weeks after use of water for mouthwash in patients with gingivitis are presented in Table 2.

Table 2. Aerobic and Facultative Bacterial Counts Before and Two Weeks After Use of Water for Mouthwash in Patients with Gingivitis.

|  |  |  |
| --- | --- | --- |
|  | Before | After Two Weeks |
| Mean | 26.88 | 25.88 |
| Standard Deviation | 7.51 | 9.01 |
| Minimum | 18 | 17 |
| Maximum | 37 | 40 |
| Count | 8 | 8 |

 **Test for significant differences in the reduction of aerobic and facultative bacterial counts after using 0.2% CHX, PM, Listerine and water as mouthwash:**

 Analysis of Variance was used to analyze the differences in the reduction of aerobic and facultative bacterial counts after using 0.2% CHX, PM, Listerine and water rinses.

 The results showed that the mean bacterial count after using 0.2% CHX for two weeks decreased by 23.13. This was followed by Listerine®, with a mean reduction of 19.75. PM resulted in 13.5 decrease in the mean bacterial count, while water reduced the bacterial count by only 1.

The above observations are further illustrated in Figure 1.

Figure 1. Bacterial Count Reduction after Two Weeks Use of 0.2% CHX, Listerine, PM, and Water.

 C1-C2 Amount of the mean total bacterial reduction after use of 0.2% CHX

 L1-L2 Amount of the mean total bacterial reduction after use of Listerine®

 P1-P2 Amount of the mean total bacterial reduction after use of PM

 W1-W2 Amount of the mean total bacterial reduction after use of water

 It can be observed in Figure 1 that 0.2% CHX caused the highest reduction in bacterial counts, followed by Listerine® and PM. However, water was ineffective in reducing bacterial counts. These findings show that the three mouthwashes tested in this study may be effective in reducing bacterial count in the oral cavity after two weeks.

 Subsequent ANOVA revealed that highly significant differences existed regarding the reduction capability on bacterial counts between the three mouthwashes and water. This is shown by the p-value of 6.74 x 10-9, which is lower than 0.01. To determine which means are significantly different from each other, Tukey-Kramer post hoc test was employed and the results are summarized in Table 3.

Table 3. Tukey-Kramer Mean Comparisons of the Reduction of Bacterial Counts.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Comparison | AbsoluteDifference | Std. Errorof Difference | CriticalRange | **Results** |
| 0.2% CHX vs. Listerine | 3.375 | 1.77831054 | 8.5359 | **Insignificant** |
| 0.2% CHX vs. PM | 9.625 | 1.77831054 | 8.5359 |  **Significantly different** |
| 0.2% CHX vs. water | 22.125 | 1.77831054 | 8.5359 |  **Significantly different** |
| Listerine vs. PM | 6.25 | 1.77831054 | 8.5359 | **Insignificant** |
| Listerine vs. Water | 18.75 | 1.77831054 | 8.5359 |  **Significantly different** |
| PM vs. Water | 12.5 | 1.77831054 | 8.5359 |  **Significantly different** |

 Figure 1 shows that 0.2% CHX was slightly more effective than Listerine® in reducing bacterial counts after two weeks of use as mouthwash although the former was slightly higher than the latter, the difference turned out to be insignificant (Table 6). In agreement with the results previously shown, 0.2% CHX mouthwash was significantly more effective than PM and water. Interestingly, the bacterial reduction was not statistically significant between Listerine® and PM. The mean bacterial count after two weeks of using Listerine® was reduced by 19.75 compared to 13.5 for PM.

The antibacterial effect of 0.2% CHX was significantly higher than that of PM and water but the differences between the antimicrobial effects of 0.2% CHX and Listerine® and also PM and Listerine® were not significant.

**The results of ZOI test for each mouthwash:**

 The ZOI test demonstrated bacterial growth to the space of the plate in Listerine®, PM and water groups after 24 hours. However, CHX prevented the growth of bacteria in all 32 plates. To be more specific, the diameter of the zone of inhibition, where there was no bacterial growth, was measured and the results are summarized in Table 4.

Table 4. Diameter of the Zone of Growth Inhibition by 0.2% CHX.

|  |  |
| --- | --- |
|  | Diameter of Growth Inhibition Zone (mm) |
| Mean | 18.38 |
| Standard Deviation | 6.08 |
| Minimum | 12 |
| Maximum | 27 |
| Count | 32 |

 Table 4 shows that 0.2% CHX inhibited the growth of bacteria to an average diameter of 18.38 mm with a standard deviation of 6.08. The smallest zone without bacterial growth had a diameter of 12 mm while the largest zone had a diameter of 27 mm. The results indicated a discrepancy in the demonstrated antibacterial effects of Listerine® and PM because while both mouthwashes showed bacterial count reduction after two weeks of use, they were not able to produce ZOIs after 24 hours of incubation. This can be explained by the fact that for the ZOI tests, the culture medium was treated with the mouthwashes only once and then the results were read after 24 hours; whereas, for the in vivo tests, bacteria in the oral cavity were constantly exposed to the effects of the mouthwashes for two weeks. This may mean that constant exposure to the mouthwashes is necessary to reduce bacterial counts, especially with the use of Listerine® and PM.

 Based on the findings, it is confirmed that there was a significant difference in bacterial count before and after use of 0.2% CHX, Persica, and water in patients with gingivitis; however, there was no significant difference in bacterial count before and after use of 0.2% CHX and Listerine in patients with gingivitis. ZOI test for the three mouthwashes and water showed a significant difference. Therefore, the alternate hypothesis was confirmed.

**Discussion**

 The medium used in this study was Mueller Hinton Agar, which is used in procedures commonly performed on aerobic and facultative anaerobic bacteria (neogen.com) because it is growth-specific to those bacteria. It would seem logical to assume, therefore, that the bacteria cultivated on the agar plates were indeed both aerobic and facultative.

 In this study, due to limitations of the laboratory, the ZOI could only be checked after 24 hours of incubation. It seems very probable that Listerine® and PM had lost their antibacterial effect within that time allowing for bacterial growth in the plates during incubation. The antibacterial effect of Listerine® mostly comes from its alcohol content which may have evaporated by the time the ZOI was measured. As for PM further studies need to be conducted to determine whether or not, like Listerine®, its main antibacterial component loses its effectiveness within a relatively shorter time than CHX. Therefore, in this study, the short-term antibacterial effect of Listerine® and PM could not be determined. Only CHX had antibacterial effect even after 24 hours. Because of the results presented earlier regarding the reduction in bacterial counts for both Listerine® and PM, it may be reasonable to assume that they could have produced a measurable ZOI during the first few hours of incubation. This assumption is further based on a study by Almas et al, (2005) which showed that PM had antibacterial activity although much lower compared to CHX. What is apparent from the results of this study is that CHX has more longevity than Listerine® or PM.

 It is difficult to remove all bacteria by mechanical plaque control; thus, antibacterial mouthwashes can be useful adjuncts for this purpose. In the current study, the antibacterial effects of three mouthwashes were compared in vitro and also in vivo. ZOI test (in vitro) was used for supragingival plaque samples of 32 patients with gingivitis before using mouthwashes while CFU test (in vivo) was used for these patients before and two weeks after using the mouthwashes. All three commercial mouthwashes (0.2% CHX, Listerine® and PM) turned out to be more effective than water in reducing bacterial counts after two weeks of use as mouthwash. Although the 0.2% CHX was slightly more effective than Listerine®, the difference turned out to be insignificant. PM was also effective in reducing bacterial counts but to a significantly lesser degree than 0.2% CHX. These findings were similar to those of Salehi and Momeni Danaie (2006)(4) and Tomas et al, (2008)(11) who found that the bacterial count in the oral cavity decreased after using CHX mouthwash. Their results showed a significant reduction in the total bacterial population at 30 seconds and one hour after mouthrinsing with both CHX concentrations of 0.12% and 0.2%; also, CHX had the highest antimicrobial effects in orthodontic patients.

 Kasuga et al, (1997)(12) also demonstrated a reduction in bacterial counts after using Listerine® mouthwash. They reported that mouth washing with Listerine® for 30 seconds resulted in a decrease of the viable bacterial counts in the saliva. Al-Bayati and Sumaiman’s (2008)(13) study also showed total bacterial reduction after using PM. They reported that the strongest antibacterial activity was observed using the aqueous extract of Salvadora persica against *S. faecalis*.

 Similarly, Balbuena et al. (1998)(14) demonstrated that both mouthwashes (CHX and Listerine®) significantly reduced bacterial counts one hour after treatment in their volunteers and at 4 hours after treatment and Peridex® (CHX) oral rinse showed a further reduction in the bacterial colony count.

 Our findings confirmed that there was a significant difference in bacterial count before and after using 0.2% CHX, Persica, and water in patients with gingivitis; however, there was no significant difference in bacterial count before and after using 0.2% CHX and Listerine in patients with gingivitis. ZOI test for the three mouthwashes and water showed a significant difference. Therefore, the alternate hypothesis was confirmed.

**Conclusion**

 The results simply revealed that mouthwashes are necessary to maintain low bacterial counts in the mouth. However, since the beneficial role of the presence of commensal species in the oral cavity has been established, the need to constantly maintain low bacterial counts in the mouth is still under debate. The results obtained from this study revealed that the three mouthwashes may decrease the number of bacteria in the oral cavity.

**Recommendations:**

 It is recommended that further studies be conducted on the effect of different mouthwashes on not only the aerobic and facultative bacteria but also on anaerobic bacteria in the oral cavity. It is also recommended to increase the time period of using the mouthwashe~~s~~ by each patient. Moreover, it would be beneficial to test the efficacy of mouthwashes in patients with chronic periodontitis or aggressive periodontitis as well as testing the actual mechanism of each mouthwash against bacteria recolonization. A ZOI test evaluation after 1 hour, 6 hours, 12 hours, 18 hours and 24 hours is also recommended.

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