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Effect of a Combination of Photodynamic Therapy and Chitosan on *Streptococcus mutans* (An In Vitro Study)

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Abstract

Introduction: This study aimed to assess the effect of photodynamic therapy (PDT) and chitosan separately and in combination on *Streptococcus mutans*.

Materials and Methods: This in vitro experimental study evaluated 216 microbial samples in 6 groups. First, 5 µL of 0.5 McFarland standard suspension of *S. mutans* was added to each well of an ELISA microplate; 100 µL of Mueller Hinton broth was also added to each well; 180 wells contained *S. mutans* suspension while 36 wells were devoid of bacteria. Group 1 served as the negative control and had no bacteria. Group 2 served as the positive control and *S. mutans* in the positive control wells did not undergo any intervention. In groups 3 and 4, PDT with a 50 mW low-level laser was performed for 30 and 40 seconds respectively. In group 5, 3 mg/mL of chitosan (100 µL) was used. In group 6, 3 mg/mL (100 µL) of chitosan was used in combination with PDT (50 mW laser for 30 seconds). The laser was irradiated under aseptic conditions at a 660 nm wavelength with 50 mW power. Data were analyzed using one-way ANOVA and Tukey's test.

Results: PDT combined with chitosan showed maximum bactericidal effect followed by PDT for 40 seconds and chitosan groups ($P<0.05$). PDT for 30 seconds showed a minimum bactericidal effect ($P<0.05$). All pairwise comparisons revealed significant differences ($P<0.001$).

Conclusion: Chitosan and PDT alone can be used to decrease the *S. mutans* count. However, their combined use has a greater bactericidal effect on *S. mutans*.

Keywords: *Streptococcus mutans*; Chitosan; Photodynamic therapy.

Introduction

Despite the advances in caries control programs and increased use of fluoride, the prevalence of dental caries is still high worldwide.[1,2] Dental caries can cause pain and affect patients' quality of life.[3,4] Dental caries has a multifactorial origin and is caused by the interaction of some internal factors such as decreased salivary flow, tooth surface morphology, poor nutritional status and hormonal conditions and external factors such as microbial flora, poor oral hygiene and low access to fluoride.[5]

Biofilm formation is a biological process mediated by the attachment of oral bacteria to tooth surfaces and their proliferation. Dental biofilm forms following the adhesion of planktonic bacteria to the pellicle covering the tooth surface.[6] Only limited bacteria can attach to tooth surfaces such as *Streptococcus mutans* and lactobacilli.[7,8] *S. mutans* is one of the main causes of dental caries and is the target of the majority of preventive strategies.[9] Prevention is the most efficient method of caries control.[10] Several antimicrobial agents such as xylitol,[11] chlorhexidine,[12] fluoride [13] and chitosan have been introduced for caries prevention. Chitosan is a polysaccharide composed of glucosamine and N-acetyl glucosamine copolymers.[14] Chitosan could be synthesized by partial deacetylation of chitin. Chitosan is formed of a series of polymers that have different molecular weight, viscosity and deacetylation rates.[15] Chitosan is biocompatible and has chelating capability.[16] Chitosan can induce the nucleation of crystals on the dentin surface and enhance the mineralization of demineralized enamel by the use of calcium and phosphate. Chitosan biopolymers are used

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for the treatment of carious dentin.[17] Chitosan has strong bactericidal activity and is not toxic for mammals.[18] It has anti-tumor and wound-healing properties and is muco-adhesive.[19] It has antibacterial and antifungal properties as well.[20] Its antimicrobial properties are related to its attachment to bacterial DNA.[21] It also increases the bacterial membrane permeability and causes the leakage of cellular components. It interferes with mRNA synthesis and subsequently prevents protein synthesis.[22] The inhibitory effect of chitosan on streptococci has been previously reported.[23] Chitosan interferes with the adhesion of *S. mutans* to dental biofilm and prevents biofilm maturation.[24]

Photodynamic therapy (PDT) is a non-invasive modality that uses a low-level laser to prevent the growth and proliferation of bacteria such as *S. mutans*. It is commonly used in periodontal therapy. In PDT, visible light is used to activate a photosensitizer, which produces reactive oxygen species upon activation, causing photo-toxicity. The efficacy of PDT depends on a number of factors such as type of visible light, laser settings, and reaction with the photosensitizer.[25] Free radicals produced in PDT have toxic effects on the bacteria but not on host cells in most of the cases.[26] This minimally invasive modality is effective against resistant bacterial species. Also, it could be used in the management of solid cancers or ocular vascularization diseases.[27,28] It does not cause any unwanted damage to tissue.[29] The efficacy of photosensitizers depends on their dosage and site of application. Methylene blue is one of the commonly used materials as a photosensitizer.[30]

Several antibacterial agents such as chlorhexidine, metronidazole and quaternary ammonium compounds are used for the elimination of cariogenic microorganisms and the prevention of dental caries, but they have side effects such as staining, increasing calculus formation and causing diarrhea by changing the normal microbial flora of the gastrointestinal system.[31] Thus, there is a need for new strategies to prevent the growth and proletarian of *S. mutans* and dental caries. Studies on the use of chitosan for the prevention of caries and its effect on *S. mutans* are limited, and the available ones have reported conflicting results.

There are a large number of studies on PDT as a conservative antibacterial modality. However, they are widely variable in methodology and sample size. Therefore, it is impossible to make a definite conclusion regarding the efficacy of PDT in the elimination of cariogenic bacteria. Thus, this study aimed to assess the effects of PDT, chitosan and a combination of the two on *S. mutans*.

Materials and Methods

In this in vitro experimental study, the sample size was calculated to be 216 samples (n = 36 in each group) according to a study by Camacho-Alonso et al,[32] assuming alpha=0.05, beta=0.2 and power of 80% using PASS 2015 software.

The microorganisms used in this study were the standard strains of *S. mutans* ATCC 3198 obtained from the microbial bank of the microbiology laboratory of the School of Medicine at Shahid Beheshti University of Medical Sciences and they were cultured on blood agar. They were then incubated at 37°C for 48 hours in anaerobic conditions (CO2, H, and N) to decrease variability and confirm the phenotype of cells growth After the formation of colonies, 0.5 McFarland standard suspension of *S. mutans* was prepared. The cultures that were incubated were then homogenized with a vortexer, and then their optical density (OD) was measured with a microplate reader on a wavelength of 600 nm. Each 1 cc of the suspension contained 1.5×10^8 bacteria. Next, 100 µL of Mueller-Hinton broth culture medium (Thermo Scientific, Waltham, MA, USA) was added to each well of an ELISA microplate (Streptavidin ELISA Plate Safety Data Sheet - SDS) (Figure 1); 5 µL of 0.5 McFarland standard suspension of *S. mutans* was also added to 36 wells as the negative control. These wells were devoid of bacteria. The study groups were as follows:

Group 1 served as the negative control and was devoid of bacteria. Group 2 served as the positive control and the wells contained *S. mutans* that did not undergo any intervention. In group 3, PDT with a low-level laser with 50 mW power was performed for 30 seconds (1.5 J). In group 4, PDT with 50 mW power was performed for 40 seconds (2 J). In group 5, 3 mg/mL of chitosan in an amount of 100 µL was added to the wells according to a study by Camacho-Alonso et al.[32] In group 6, 3 mg/mL of chitosan (100 µL) was used in addition to PDT with 50 mW power for 30 seconds (1.5 J). Thirty-six samples were evaluated in each group.

The laser was irradiated under aseptic conditions at a 660 nm wavelength (Hager and Werken GmbH and Co. Duisburg, Germany) (Figure 2) under a laminar flow hood. The calibration is evaluated by a power meter. The spot area (laser aperture) was 0.38 cm^2. The tip of the laser handpiece was fixed in a vertical position at the opening of the well. To prevent accidental irradiation of adjacent wells and bias in the results, two empty wells were considered between the study wells. The plate was covered with a

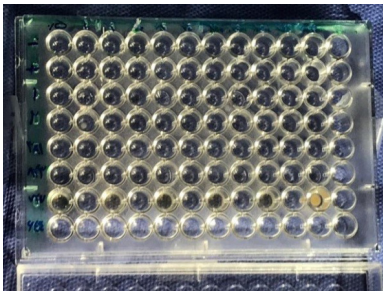

Figure 1. ELISA Microplate for Samples.

black shield that had a hole in it corresponding to the size of the opening of one well (7 mm) to prevent accidental irradiation of other wells.

Methylene blue (100 µL; Merck, Germany) in 0.01% concentration was used as a photosensitizer in PDT. Methylene blue was added to each well and was irradiated with the laser after 3 minutes (Figure 3). The interval between the laser aperture and the sample surface was fixed at 1 mm in all study groups.

Chitosan paste with low viscosity (Merck, Germany) was dissolved in 3 mg/mL of 1% acetic acid (Merck, Germany) and was added to each well in an amount of 100 µL.

After the interventions, 0.02 cc of the contents of each well was removed by a sterile loop and cultured on the blood agar culture medium (Figure 4). They were incubated at 37°C for 24 hours. After incubation, the number of colony-forming units per milliliter (CFU/mL) was determined.

Normal distribution of data was evaluated using the Kolmogorov-Smirnov test. The homogeneity of variances was evaluated using the Levene's test. The groups were compared using one-way ANOVA. Pairwise comparisons were carried out using the Tukey's HSD test. The level of significance was set at 0.05. All statistical analyses were carried out using Excel 2013 software.

Results

The Kolmogorov-Smirnov test confirmed the normal distribution of data in all groups ($P>0.05$). The homogeneity of variances was also confirmed by the Levene's test ($P>0.05$). Non-treatment groups (negative and positive control groups) were chosen to standardize the study protocol. The s. mutans count was zero in the negative control group. The positive control group showed the highest number of CFU/mL.

Table 1 shows the number of colonies in the four treatment groups, indicative of the bactericidal effects of the four modalities. One-way ANOVA showed a significant difference in the bactericidal effects of the four groups ($P<0.001$). Thus, pairwise comparisons of the groups were performed using the Tukey's HSD test. The results showed a maximum bactericidal effect in the combination of PDT and chitosan followed by PDT for 40 seconds and chitosan groups. PDT for 30 seconds showed a minimum bactericidal effect (Figure 5). All pairwise comparisons yielded significant differences ($P<0.001$ for all six comparisons, Table 2).

Figure 2. Photodynamic Therapy Device.

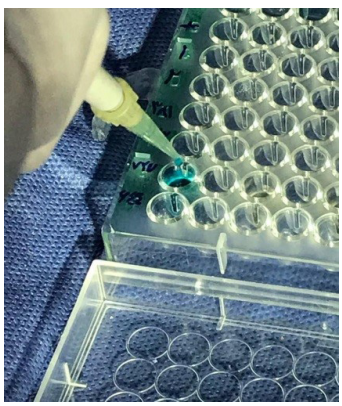

Figure 3. Methylene Blue Added to Each Well.

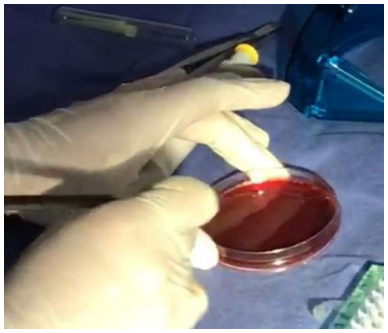

Figure 4. Blood Agar Culture.

Discussion

This study assessed the effect of PDT, chitosan and a combination of the two on *S. mutans*. The results revealed that PDT plus chitosan had maximum bactericidal effect followed by PDT for 40 seconds and chitosan groups. A minimum bactericidal effect was noted after PDT for 30 seconds. All pairwise comparisons yielded significant differences ($P<0.001$).

Zheng and Zhu[33] discussed that the mechanism of action of chitosan in the inhibition of bacterial growth is based on modifying the bacterial cell wall and permeability of the cell membrane. The addition of this biopolymer to toothpaste can prevent the attachment of *S. mutans* to tooth surfaces.[34] Abedian et al[35] found that chitosan can inhibit the growth and proliferation of *S. mutans*. Also, it prevents biofilm formation on tooth surfaces. Costa et al.[36] evaluated mouthwashes containing chitosan and concluded that it can decrease the count of *S. mutans*. Chen and Chung[37] Fujiwara et al,[38] Kawakita et al,[39] and de Paz et al[40] used chitosan dissolved in water as a mouthwash against *S. mutans* and found similar results. Hayashi et al[23] evaluated the effect of chitosan chewing gum on *S. mutans* and reported the growth

Table 1. Number of Colonies in the Four Experimental Groups, Indicative of the Bactericidal Effects of the Four Modalities (n=36)

Group	Maximum CFU/Ml	Minimum CFU/Ml	95% CI		Standard Deviation	Mean
			Upper Bound	Lower Bound		
PDT for 30 seconds	3000.00	2100.00	2560.5859	2431.0808	191.37659	2495.8333
PDT for 40 seconds	1350.00	900.00	1175.9403	1090.7264	125.92515	1133.3333
PDT with chitosan	750.00	200.00	440.0700	346.0411	138.95157	393.0556
Chitosan alone	2250.00	1500.00	1868.9009	1761.6546	158.48364	1815.2778

Table 2. Pairwise Comparisons of the Groups in Terms of the Colony Count (Bactericidal Effects)

Group (I)	Group (J)	Standard Error	Mean Difference	*P* Value
PDT for 30 seconds	PDT for 40 seconds	36.68688	1362.50000	0.000
	PDT with chitosan	36.68688	2102.77778	0.000
	Chitosan	36.68688	680.55556	0.000
PDT for 40 seconds	PDT with chitosan	36.68688	740.27778	0.000
	Chitosan	36.68688	-681.94444	0.000
PDT with chitosan	Chitosan	36.68688	-1422.22222	0.000

inhibition of *S. mutans* and reduction of its count in the saliva following the use of chitosan chewing gum. In the present study, chitosan significantly decreased the count of *S. mutans*, which was in line with the findings of the abovementioned studies.[23,37-40]

Previous studies on the bactericidal effects of PDT did not use the same protocol, and wide variability exists in the wavelength of light, its energy density and duration of exposure.[25-27] In the present study, 50 mW power was used and the radiation time was 30 and 40 seconds. Chitosan was used in combination with PDT in this study and yielded positive results, showing a maximum bactericidal effect on *S. mutans* due to the synergistic effect of chitosan and PDT. Fabio et al.[41] evaluated the effect of chitosan plus PDT on Candida albicans and concluded that the addition of chitosan to methylene blue did not enhance the antifungal effects of PDT. Their results were different from our findings, which may be due to the fact that different microorganisms were evaluated in the two studies. Chien et al[42] used chitosan to enhance the effects of PDT on Candida albicans and reported the optimal efficacy of this combined modality.

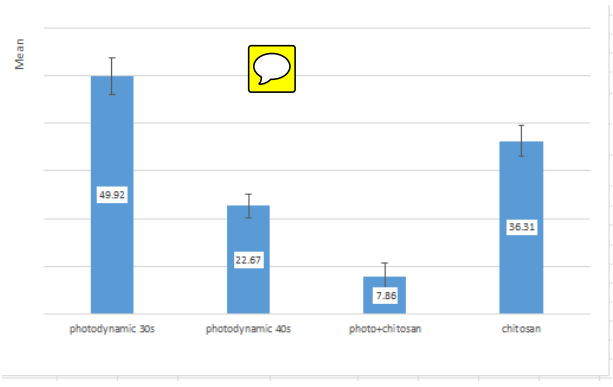

Figure 5. Comparison of the Bactericidal Effect of Different Therapeutic Methods.

The difference between their results and those of Fabio et al[41] may be due to different sample sizes. Peng et al.[43] evaluated the chitosan hydrogels to enhance the efficacy of PDT against *Staphylococcus aureus, Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* and concluded that the combination of chitosan and PDT enhanced the efficacy of PDT for the treatment of periodontal pockets; their results were in line with our findings.

Camacho-Alonso et al[32] evaluated the effect of chitosan plus PDT on *Enterococcus faecalis*, which is the main culprit responsible for treatment-resistant endodontic infections. They observed that this combination caused a greater reduction in the bacterial count compared with the use of each modality alone. This result was in agreement with ours.

Gong et al[44] assessed the effects of PDT on *S. mutans* and showed that PDT significantly decreased the *S. mutans* count, which was in line with our results. Alves et al[45] evaluated the antimicrobial effects of PDT on carious lesions of primary molars and reported that PDT can serve as an adjunct to caries removal and restoration of teeth for the reduction of the *S. mutans* count. Azizi et al[46] evaluated the effects of PDT on *S. mutans*. They used 660 and 810 nm wavelengths of laser with 100 and 40 mW power for 60 seconds and concluded that PDT can eradicate *S. mutans* colonies. Fekrazad et al[47] concluded that PDT can significantly decrease the count of *S. mutans* in children's saliva with early childhood caries immediately after the intervention. Similar results were reported by Beytollahi et al[48] and Rolim et al[49] These results were in agreement with our findings and highlighted the optimal efficacy of PDT for the elimination of *S. mutans*. We irradiated a low-level laser for 30 and 40 seconds in our study. Considering the short duration of laser irradiation in our study, this protocol can be used for pediatric patients with poor cooperation. However, most previous studies irradiated a

laser for longer periods of time for periodontal purposes in adults. Further studies are required on ideal laser parameters to achieve the best bactericidal effects. In vitro design, which limits the generalizability of the results to the clinical setting, was a limitation of this study. Future in vivo studies are required to increase the generalizability of the results to the clinical setting. Also, the efficacy of higher concentrations of chitosan should be evaluated in future studies.

Conclusion

Chitosan and PDT can be used as alternative modalities for the reduction of the *S. mutans* count. Also, a combination of the two showed a greater bactericidal effect on the *S. mutans* count. Thus, considering its non-invasiveness, this innovative combination technique can be used to control caries in pediatric patients.

Ethical Considerations

This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1397.026).

Conflict of Interests

The authors declare that they have no conflict of interest.

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