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Reduction of *Streptococcus salivarius* by Chlorella-Mediated Antimicrobial Photodynamic Therapy

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Abstract

Introduction: Nowadays, antimicrobial photodynamic therapy (aPDT) has been introduced as one of the minimally invasive methods for disinfection of the surfaces of dental implants. Being derived from seaweed, Chlorella has been used as a photosensitizer in this study. This study aimed to investigate the impacts of aPDT with Chlorella on the rate of reduction of *Streptococcus salivarius* in vitro.

Methods: The minimum inhibitory concentration of Chlorella, the sublethal exposure to 660 nm diode laser irradiation, and the minimum sublethal dose of aPDT utilizing Chlorella against *S. salivarius* were determined. Finally, the CFU/mL value of each plate was calculated. Then, Tukey HSD and one-way ANOVA tests were utilized for comparison the number of colonies after the interventions.

Results: A concentration of 250 µg/mL of Chlorella at an irradiation time of 3 minutes, was identified as a sublethal dose of aPDT for the reduction of *S. salivarius*. In contrast, the application of aPDT utilizing a 660 nm diode laser for 4 minutes in combination with Chlorella at a final concentration of 500 µg/mL, demonstrated significantly greater efficacy in reducing *S. salivarius* compared to the other experimental groups ($P<0.001$).

Conclusion: Chlorella 500 µg/mL mediated aPDT (660 nm, 4 minutes) has a significant effect on reducing *S. salivarius* count.

Keywords: *Streptococcus salivarius*; Antimicrobial photodynamic therapy; Chlorella; Peri-implant diseases.

Introduction

Peri-implant diseases are to inflammatory conditions that affect the hard and soft tissues of the gingiva around dental implants. Similar to natural teeth, bacterial colonies can form and multiply on the implant surface and below the gum line. It can releases toxins and inflammatory mediators that can damage gingival tissues, trigger inflammation, and accelerate alveolar bone loss, potentially leading to peri-implantitis.

According to the studies, the sulcus microbiota around the implant with healthy or mucositis is very similar to the healthy gingival microbiota or gingivitis. Conversely, the microbiota detected in peri-implant infection is in most cases - but not all - similar to the periodontal pockets in teeth with advanced periodontitis. With the increasing use of dental implants, the prevalence of complications from this treatment has also increased. Peri-implantitis affects the tissues around an osseointegrated implant and is accompanied by deep pockets, infection, and loss of marginal bone around the implant.[1,2]

The bacteria associated with diseases related to the implant are nearly similar to ones found in periodontitis, such as *Streptococcus salivarius*, *Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis.*[3] Key strategies for addressing peri-implantitis include the elimination of bacterial biofilm while minimizing harm to the implant surface, the detoxification of bacterial byproducts, and the management of inflammation. Inadequate and untimely treatment of such diseases can lead to complications, including bone deterioration, discomfort, and implant loosening. Ultimately, this may necessitate the removal of the implant by the dentist, resulting in numerous challenges for both dental professionals and patients.[4]

Recent research has suggested the use of laser disinfection and antimicrobial photodynamic therapy (aPDT) as potential treatments for peri-implantitis. Nevertheless, the significant expenses associated with lasers and the considerable dimensions of the equipment present challenges. Additionally, high-power lasers may

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lead to unintended temperature rises at the target site.[5] aPDT is used to kill microorganisms that cause caries and periodontal disease. This treatment is based on three principles: photosensitizer, light source and oxygen.[6,7]

This method is characterized by its non-invasive nature and renewability. Furthermore, it exhibits a high degree of specificity in targeting bacteria and presents a minimal risk of developing bacterial resistance following its use.[8] Given that this treatment relies on oxygen, there are constraints concerning its use at infection sites that have minimal or no oxygen available.[9] The photosensitizer is also stimulated by applying light at specific wavelengths, causing a chemical reaction at the site and releasing radical oxygen, which is a toxic agent for cells in the area.[10]

Streptococcus salivarius is a gram-positive, facultatively anaerobic bacterium.[11] It was the first bacterium to be colonized in the oral cavity in the first hour of birth. This bacterium was present in all oral samples. The growth rate of these bacteria in dynamic and static samples was not significantly different. The growth impact of *S. salivarius* and other species of *Streptococcus* on implant wear was studied, indicating more corrosion-wear performance in *S. salivarius*.[11]

Chlorella as a natural photosensitizer isolated from seaweed has high efficiency and low toxicity properties and can be used as a functional food.[12] Chlorella is used as a health supplement mainly in the United States and Canada and as a dietary supplement in Japan.[13]

Due to the increasing use of implant therapy and the associated increase in the prevalence of peri-implantitis that causes treatment failures and the role of *S. salivarius* in such diseases, an effective method to quantitatively reduce this bacterium is required.[14-16]

Chlorella as a new photosensitizer has recently been introduced to the dental market. Therefore, very limited research has been conducted on the effect of this substance on peri-implant diseases. Furthermore, there was no information about the antimicrobial properties of Chlorella on *S. salivarius* in the previous studies. In light of this information gap, the current research was conducted to assess the impact of aPDT utilizing Chlorella on the in vitro disinfection of *S. salivarius*.

Materials and Methods

The bacterium examined, *S. salivarius* (ATCC 7073), was obtained from the American Type Culture Collection (Manassas, VA, USA). The strain was grown in brain-heart infusion (BHI) broth (Ibresco, Iran) at 37 °C with 5% carbon dioxide (CO_2) to match 0.5 McFarland standard (OD_{625} = 0.08-0.1) corresponding to 1.5×10^8 CFU/mL, which was considered as 100× inoculum.[17]

Determining Sample Size

For sublethal concentration and irradiation time, we repeated each experiment 3 times including 3 samples in each. One-way ANOVA power analysis was applied by using PASS 11 software to determine the sample size considering $\alpha = 0.05$ and $\beta = 0.2$. The mean of the colony count was 77×10^4, with an effect size of 0.75. Consequently, the minimum sample size necessary for each of the four groups was determined to be six samples.

Photosensitizer and Light Source

Chlorella (Weber Medical, Germany) was dissolved in distilled water to create a fresh stock solution with a concentration of 4000 µg/mL. The solution was stored in a dark environment at room temperature before utilization. A 660 nm diode laser (Konftec, Taiwan) with a power output of 150 mW was employed to activate Chlorella. The power density of the device was 0.25 w/cm^2. The laser power was verified by a power meter (Coherent, USA) prior to conducting the experiments.

Determining the Minimum Inhibitory Concentration

In order to obtain the MIC of Chlorella against *S. salivarius*, an addition of 100 µL of 2X BHI broth culture medium was made to each well of the 96-well round bottom microplate. Then 100 µL of Chlorella at a concentration of 4000 µg/mL was poured into the wells of the first column, and after that, 1/2 dilution was conducted to produce concentrations of 3.9, 7.8, 15.6, 32.1, 62.5, 125, 250, 500, 1000, and 2000 µg/mL. One hundred microliter of *S. salivarius* suspension in a concentration of 1.5×10^6 CFU/mL was then added to each well. After incubation in a partial atmosphere of 5% CO2 for 24 hours, 10 µL from the suspension collected from each well was introduced into a well of a 96-well microplate that contained 90 µL of BHI broth culture medium. Following five successive dilutions, 10 µL from each well was inoculated onto BHI agar plates (Merck, Darmstadt, Germany) for spreading culture. The plates were incubated at 37 °C with 5% CO_2 for 48 hours, after which the CFU/mL for each sample was determined by using the Miles and Misra method.[18] Wells containing *S. salivarius* suspension and wells containing BHI broth culture medium without *S. salivarius* or Chlorella suspension were respectively considered as positive and negative controls.

Determining the Minimum Lethal Time of 660 nm Diode Laser Radiation

To ascertain the minimum lethal exposure duration of laser treatment on *S. salivarius*, 200 µL of *S. salivarius* suspension with a final concentration of 1.5×10^5 CFUs/mL was subjected to irradiation by using a 660 nm diode laser. The laser operated at a power of 150 mW, with a power density of 0.25 w/cm^2 and a tip diameter of 8 mm, for intervals of 1, 2, 3, 4, and 5 minutes at ambient temperature. Following the irradiation process, the plates were incubated at 37 °C for a period of 24 hours, after which the minimum lethal dose of the laser was evaluated based on the colony count.[18]

Determining the Minimum Lethal Dose of aPDT

In order to determine the sublethal dose of aPDT, 100 µL of 2X BHI was added to each well of a 96-well flat-bottom microplate and then 100 µL of Chlorella at a concentration of one-half of the amount of MIC was poured into the first well. It was periodically diluted to a concentration of 1/8 MIC. Subsequently, 100 µL of a fresh suspension of *S. salivarius* was introduced into each well, achieving a final concentration of 1.5×10^5. The sample was then transferred to the microplate. The microplate was incubated in a dark environment at room temperature for 5 minutes. Following this, the suspension was subjected to a sublethal dose of 660 nm diode laser irradiation. The plates were then incubated at 37 °C in a 5% CO_2 atmosphere for 24 hours, and the sublethal dose of aPDT was determined based on the methods mentioned above.

Antimicrobial Photodynamic Therapy With MIC of Chlorella

It was randomly assigned to two case groups and two control groups, designated as positive and negative controls.

1. Negative control group: the bacterial suspension was not treated at all.
2. Positive control group: the bacterial suspension was subjected to 0.2% chlorhexidine for 5 minutes.
3. The bacterial suspension was subjected to Chlorella with a concentration of 500 µg/mL for 5 minutes
4. The bacterial suspension was subjected to aPDT (500 µg/mL of Chlorella for 5 minutes + 660 nm diode laser (4 minutes)).

Statistical Analysis

Statistical analysis of the data obtained in each section was analyzed by SPSS 23 software (SPSS Inc., Armonk, New York, USA). Initially, the t-test and one-way ANOVA were employed to evaluate the significance of the differences between the groups. Subsequently, a supplementary post hoc test was conducted to identify the specific differences between the two groups. A *P* value of less than 0.05 was deemed significant.

Results

MIC of Chlorella

The findings initially showed that Chlorella reduced the growth of *S. salivarius*. As shown in Figure 1, Chlorella at concentrations of 500 to 2000 µg/mL reduce the number of bacteria compared to the control ($P < 0.05$). However, no significant decrease was observed in the number of bacteria killed at a concentration of 3.9 to 250 µg/mL of Chlorella ($P > 0.05$). Therefore, the concentration of 500 µg/mL was considered as MIC.

Sublethal dose of the 660 nm Diode Laser

The results indicated that when considering the duration of laser irradiation, exposure for a period of up to 5 minutes likely does not result in a total loss of *S. salivarius* growth. Nevertheless, the 5-minute irradiation duration led to a significant decrease in the bacterial count when compared to shorter exposure times and the control group ($P < 0.05$). Nevertheless, the time of radiation under 5 minutes caused no significant difference ($P > 0.05$). Therefore, 4 minutes was considered as the sublethal dose of the 660 nm diode laser on this microorganism (Figure 2).

Sublethal Dose of aPDT

The application of aPDT utilizing Chlorella at concentrations of 125 and 250 µg/mL, along with an irradiation duration of 4 minutes, demonstrates a significant impact on the reduction of *S. salivarius* ($P < 0.05$). Consequently, a concentration of 250 µg/mL combined with a 3-minute exposure time was identified as the sublethal dose of aPDT with Chlorella for this bacterial strain (Figure 3).

Minimum Lethal Dose of aPDT

The aPDT group, which consisted of 500 µg/mL of Chlorella combined with a 660 nm diode laser for 4 minutes, demonstrated a notable decrease in the microbial count when compared to both the Chlorella group alone and the control group ($P < 0.01$). However, there was no significant difference observed in the reduction of *S. salivarius* between the control group and the Chlorella group alone ($P = 0.987$) (Figure 4).

Discussion

This study was performed to assess the effect of aPDT using Chlorella and 660 nm diode laser on *S. salivarius* in vitro. The findings indicated that the aPDT notably decreased the bacterial count. The rate of bacterial reduction in the group of aPDT was 69%, which was the highest reduction in CFU/mL compared to other groups (except for chlorhexidine). However, complete bacterial clearance was observed only in the chlorhexidine group. Nevertheless, chlorhexidine had a cytotoxic effect on host cells. As Giannelli et al[19] showed in a study when the surfaces of chlorhexidine-impregnated titanium disks were washed prior to culture, there were signs of cytoplasm and mitochondrial damage as well as apoptosis of the nucleus of macrophages around the disc; nevertheless, macrophages adjacent to the discs treated with aPDT were in normal condition and had only very little inflammation in their mitochondria.

Eick et al stated that aPDT using LED and toluidine blue is effective in periodontal pathogens but is not able to remove the biofilm completely.[20] Schär et al concluded in their research that laser irradiation time, wavelength, and light absorption by bacteria can be important and influential factors in the effectiveness of aPDT technique.[21]

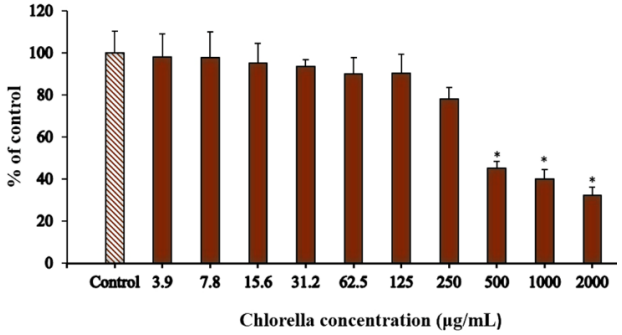

Figure 1. Determining the MIC of Chlorella

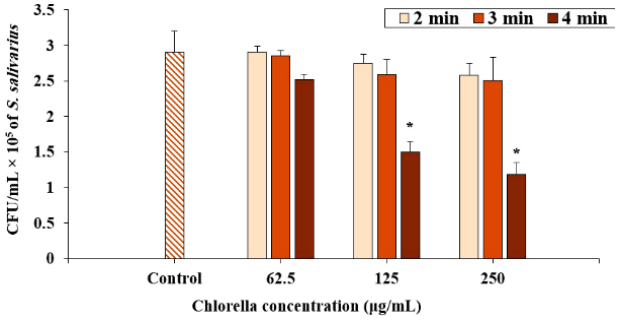

Figure 3. CFU/mL of *Streptococcus salivarius* After Different Doses of aPDT

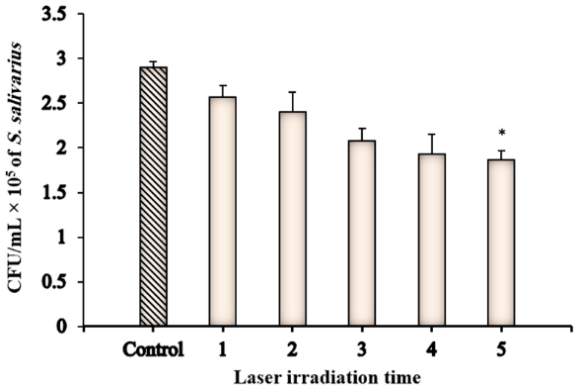

Figure 2. Effect of Different Irradiation Times of a 660 nm Diode Laser on CFU/mL of *Streptococcus salivarius*

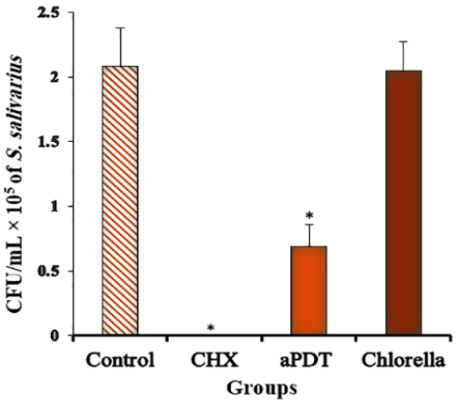

Figure 4. CFU/mL of the Study Groups

In a research study carried out by Al-Akhras et al, Chlorella was first studied as a photosensitizer in the aPDT. The maximum absorption in Chlorella was 675 nm.[22] In another study,[23] the maximum uptake of Chlorella was announced as 67 2±10nm. This study investigated the effect of disinfection by a light source at a wavelength of 660 nm with Chlorella on *Enterococcus faecalis* inside the tooth root; the cases were divided into 7 groups. Then it was found that the highest disinfection effect of *E. faecalis* was observed in the fifth group, that is, the group with 4 times the concentration of Chlorella along with the laser, and the lowest disinfection effect was observed in the third group, that is, the laser group without Chlorella. Different concentrations of MIC Chlorella significantly killed the microorganism *E. faecalis* in this study, but in the present study, different concentrations of MIC Chlorella alone did not significantly reduce the number of *S. salivarius*.

S. salivarius is also one of the most effective bacteria in periodontal disease. A study conducted by Figueiredo-Pina et al concluded that *S. salivarius* was the first bacterium to be found in the oral cavity.[11] Among the published articles, no research directly examines the extent of aPDT with Chlorella on *S. salivarius*, and this is because Chlorella has recently been introduced as one of the photosensitizers.[24] Few studies have used Chlorella as a photosensitizer substance. Marotti et al investigated the effect of aPDT with methylene blue on the implant surface. Their study suggested aPDT as an effective way to reduce bacteria on implant surfaces. The findings of this research regarding the impact of aPDT on decreasing the prevalence of microorganisms align with the current study; however, the photosensitizer in Marotti and colleagues' study is methylene blue, but the substance used in the present study is Chlorella. In addition, their study was performed on implant surfaces, but the present study was performed on culture medium.[14] In another study, Mattiello et al., examined the effect of aPDT on *Streptococcus sanguinis* and *A. actinomycetemcomitans* in vitro. The findings of this study indicate that aPDT significantly reduces the number of *A. actinomycetemcomitans* and *S. sanguinis*. The findings of this study are consistent with the current study.[25] Also, Kim et al. who utilized aPDT with methylene blue and red diode laser concluded that the application of aPDT resulted in a notable decrease in microorganisms when compared to the use of either laser treatment alone or a photosensitizer.[26]

Hwang et al conducted a study on the aPDT using Chlorella and Curcuma against *Streptococcus mutans* biofilms. In general, the utilization of 405 nm light and either Chlorella or Curcuma as a photosensitizer demonstrated notable antimicrobial properties against *S. mutans* biofilms.[27] Future studies are needed to assess different photosensitizers on various oral microorganisms to find the best protocol for controlling oral microbial infections.

Conclusion

Considering the limitations and results of the research, it appears that chlorella-mediated aPDT has a significant effect on reducing the rate of *S. salivarius* in the laboratory and may be a suitable, cheaper and safer complementary method for disinfecting implant surfaces due to lower invasion than other methods.

Authors' Contribution

Conceptualization: Nasim Chiniforush, Ardavan Etemadi.
Data curation: Mohammad Reza Karimi.
Formal analysis: Navid Pourahmad.
Investigation: Shima Afrasiabi.
Methodology: Shima Afrasiabi, Nasim Chiniforush, Ardavan Etemadi.
Project administration: Nasim Chiniforush, Ardavan Etemadi.
Resources: Mohammad Reza Karimi.
Software: Navid Pourahmad.
Supervision: Shima Afrasiabi.
Validation: Shima Afrasiabi.
Visualization: Mohammad Reza Karimi.
Writing–original draft: Navid Pourahmad.
Writing–review & editing: Nasim Chiniforush, Shima Afrasiabi.

Ethical Approval

The study was approved by the ethics committee of Tehran Medical Sciences, Islamic Azad University, Tehran, Iran (IR.IAU.DENTAL.REC.1399.209).

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