International Journal of Scientific Research in Dental and Medical Sciences

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The Comparison of the Effect of Photodynamic Therapy Using two Photosensitizer Indocyanine Green and Methylene Blue on the Colony Count of Staphylococcus Aureus (In Vitro)

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ARTICLE INFO

Article history:
Received 28 May 2019
Received in revised form 17 June 2019
Accepted 21 June 2019
Available online 24 June 2019

Keywords:
Chlorhexidine
Indocyanine Green
Methylene Blue
Photodynamic Therapy
Staphylococcus Aureus

ABSTRACT

Background and aim: Contamination of microorganisms, including Staphylococcus Aureus, in oral saliva and oral tissues, is a common problem. Therefore, in this study, photodynamic therapy's effect on the number of oral Staphylococcus Aureus colonies. Was evaluated using two indices of Indocyanine Green and Methylene Blue with sensitizer with Chlorhexidine mouthwash.

Materials and methods: In the first stage, a new culture of ATCC St.Aureus 25923 was performed. Colonies of Staphylococcus Aureus were counted. The teeth were randomly divided into four groups: the first group was Methylene Blue, and the second group was subjected to 2% Indocyanine Green. All samples from both groups were sampled before laser irradiation and colonized in the culture medium for 24 hours. The third group of teeth was immersed in 2% Chlorhexidine mouthwash and sampled. The fourth group was considered as the control group. Also, Post hoc analysis was used for comparing before and after treatment in each group.

Results: This study showed that all three experimental groups reduced the number of Staphylococcus Aureus colonies. However, Indocyanine Green and Methylene Blue did not significantly decrease the number of colonies before and after treatment ($p > 0.05$), but Chlorhexidine caused a significant decrease in the number of Staphylococcus colony Aureus ($p < 0.05$).

Conclusion: The results of this study showed that all three groups of Chlorhexidine, Indocyanine Green and Methylene Blue, reduced the colony count of Staphylococcus Aureus, although the effects of the reduction of Staphylococcus Aureus were significantly more severe than Chlorhexidine.

1. Introduction

Staphylococcus Aureus is a colonized bacteria in the mouth and can cause oral diseases such as caries and periodontal disease.[1,2] Mouthwashes are auxiliary controls for plaque and reduce oral microorganisms, which use them along with the main mechanical methods of plaque control, which have different effects on oral tissues.[3] Mouthwashes have different effects with different compositions, which, along with their side effects, cause their intake constraints. One of the most famous is the Chlorhexidine mouthwash, which, despite its beneficial effect, has a wide range of anti-microbial effects, such as a change in the taste sensation, staining on dental surfaces and repair.[4] Nowadays, a new photochemical approach to eliminating microorganisms, called photodynamic therapy, has been of great interest.[5] The first in 1904, photodynamic therapy was used in the treatment of cancer by von Tappeiner, Iodlbauer.[6] This method combines non-toxic chemical elements (photosensitizer) and low-power optical energy, which results in the release of radicals and the effect of cationic toxins on target cells.[7] In this method, which is a non-invasive method for eliminating microorganisms, only the cells that are absorbed into the photosensitizer are destroyed.[8] However, none of the previous studies have investigated the effect of photodynamic therapy on the reduction of Staphylococcus Aureus in infected dental specimens. Therefore, in this study, we will consider photodynamic therapy's effect with two Methylene Blue and Indocyanine Green photosensitizers on Staphylococcus Aureus in infected dental specimens in the laboratory of the microbiology of Shahid Beheshti Medical School in 2015-2017. Staphylococci do not have flagella, so they are not mobile. The bacteria do not produce endospores. The bacteria that do not contain spores are more resistant to environmental conditions than other bacteria. The capsule also facilitates the attachment of bacteria to the

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https://doi.org/ 10.30485/ijsrdms.2019.89757

catheter and other devices such as shunting and grafting. This property is especially important in non-acute coagulase-negative staphylococci. Although only four percent of Staphylococcus Aureus strains have capsules, almost all types react with capsular antigens (four capsule-specific antigens are known today).[9] Since humanity has grasped the magical powers of enormous and unlimited energy of light, it has consistently and consistently with the growth of human knowledge, has been struggling to control part of the abilities of this magical force every day and to achieve its goals of social life And the technological development of the community. Photodynamic therapy is a technique used to treat infection, which can kill bacteria with two photochemical and photothermal effects.[10] In a Photothermal way, the high energy of the laser causes the bacteria to be destroyed. It is destroyed by photochemical treatment of the bacteria following exposure to a sensitive light source attached to the bacterium. At the beginning of the 20th century, Paul Ehrlich was able to establish the principles of anti-microbial Photodynamics by assessing the effect of aniline on anti-microbial and animal cells.[11] Photodynamic therapy generally consists of 3 parts: light, sensitive to light, and free radicals.[12] When its optimal wavelength stimulates a light-sensitive material, it is energized from the low-energy mode. The triple state's high half-life results in a reaction between the light-sensitive material and the environment and oxygen molecules in the tissues and oxygen. It produces singlet and other free radicals that cause tissue damage.[13] The produced cytotoxic products have a limited, short-lived half-life and radius. Due to the limited migration of O2 from its production site, the location of the primary cell destruction to the localization and assembly site. The location of the light-sensitive material depends on photodynamic therapy for topical applications without harming the body's cells.[14,15] The benefits of photodynamic therapy include: non-invasive, no need for antibiotic and sensory administration, and bacterial degradation in a short period of seconds.[16] The bactericidal effect of photodynamic therapy through two mechanisms:

•Damage to DNA

• Cytoplasmic membrane of bacteria

The damage to the membrane of the bacteria leads to the deactivation of the membrane's transfusion system and the plasma membrane enzymes and the membrane's permeability increases.[17,18]

Photosensitizer: A light-sensitive compound that is capable of absorbing light with a specific wavelength and converting it into useful energy. In the case of photodynamic therapy, it involves the production of lethal cytotoxic agents, such as oxygen. Hundreds of natural and artificial colors can act as a light-sensitive material in photodynamic therapy.[19] An ideal light-sensitive material should have the following characteristics: chemically pure and with well-known content or quickly passing out of the body, causing little systemic toxicity.[20, 21] Indocyanine Green and Methylene Blue, including light-sensitive light and diode laser, are the most commonly used laser. The high concentration of light-sensitive light in the vicinity of the laser results in the appropriate bacterial elimination without any side effects for host tissues. Among the essential light-sensitive materials, Dye (Methylene Blue, Toluene Blue, etc.), Perfringin, Chlorine, Furocoumarins, Xanthenes, Furocoumarins[22] is mentioned.

Indocyanine Green is light sensitive: This material is an anionic light-sensitive material activated at 810 nm in the wavelength, and the manger is oxidized to light. The ICG is combined in sodium salts with sodium iodide to 5% to optimize the composition for medical applications. Of course, there is no information about how much iodine causes allergic or anaphylactic reactions.[23]

Methylene Blue has been used to treat methemoglobinemia (such as cyanide toxicity), which has recently been addressed in the treatment of Alzheimer's. It is used to disable bacteria, viruses, and fungi. In recent years it has been used to inactivate viruses in fresh frozen plasma and has no toxic effects on humans. By attaching bacteria to the membrane structure, Chlorhexidine increases the permeability of the cell and intracellular cells to the outside of the cell and the formation of intracellular coagulation on the bacterial cell. Long-term use of this substance does not increase the microbial resistance and does not reduce its effectiveness. The unwanted effects of Chlorhexidine include inflammation of the parotid gland in some cases.[24]

2. Materials and methods

The study population: the first premolar teeth without cracks and decay were contaminated with Staphylococcus Aureus 0.5 McFarland suspension, and the number of colonies was the population that was studied. Sixty extracted, and sterile premolar teeth were contaminated with Staphylococcus Aureus 0.5 McFarland suspension and randomly divided into three groups of 20. Ten teeth were considered as control and non-intervention after contamination. Ten teeth were also examined without contamination. This number of samples was within the range of the number of samples examined in different experiments. The sampling method in this research was random and straightforward. Data collection was done by observing the microbial culture and counting the number of colony-forming units (CFU). In the first phase of the study, a new culture of ATCC St.Aureus 25923 was performed. Eighty premolar teeth were drawn sterilized without decay. The teeth were stained with Staphylococcus Aureus at a concentration of 0.5 McFarland, produced by the Mistletoe Manufacturing Company for 120 minutes. The incubators were kept at 37° C. Then, the teeth were washed with normal saline for 2 minutes. Before the start of treatment, sampling was performed using swabs, and in a culture medium at 37° C It was incubated for 24 hours. Then the number of Staphylococcus Aureus colonies was counted, and the information was recorded in Form No. 1. We randomly divided into four groups, and the first group was 20 teeth made by Methylene Blue in Germany at a concentration of 2% for 1 minute. The second group was 20 teeth, which was made to Indocyanine Green, a German merk factory. At a 2% concentration for 2 minutes, we then stripped both groups for 20 seconds with normal saline. We sampled all samples from both groups before laser irradiation and incubated in the Blag Agar culture medium at 37° C for 24 hours. Then The number of colonies was counted and recorded in the form of information. The first group was equipped with a laser with a wavelength of 660 nm and a power of 40 mW-CW and a 4.8 j/cm2 for 60 seconds, and the second group was exposed to contact with David's laser with a wavelength of 810 nm and a power of 100 mW-CW and a density of 12 j/cm2 for 60 seconds. Then, swab assisted sampling and incubated in Blag Agar culture medium at 37° C for 24 hours. The number of colonies was counted, and the information was recorded in the information form No. 2. The second group was 20 teeth in the Chlorhexidine mouthwash of 0.2%. The paradise pharmacy was immersed for 30 seconds. Then we sampled with a swab and incubated in a culture medium at 37° C for 24 hours, then agitated. The colonies were counted and recorded in information form No. 2. The fourth group was 10 positive controls (Staphylococcus Aureus suspension without coloring or Chlorhexidine) and 10 negative controls (the desired reference or Chlorhexidine without bacteria) were considered for each sample group.[26, 27, 28] Finally, after collecting the required information from all studied samples, we analyzed the data in which we used the SPSS Version 20 software. Before and after the treatment of all three groups, information of all three groups was selected according to descriptive

analysis (Descriptive statistics), and the comparison of the number of colonies between the different groups before and after the treatment was investigated according to Post Hoc analysis.

3. Results

The central dispersal indices and standard deviation of the number of Staphylococcus Aureus colonies in different groups before and after treatment were described in Table 1. This analysis showed that the number of Staphylococcus Aureus colonies after treatment in all three groups Chlorhexidine, Methylene Blue + 660 nm diode laser, and indocyanine Green + laser diode 810 nm. There was no significant difference between the groups of indocyanine Green and Methylene Blue before and after laser radiation (p> 0.05) but in the Chlorhexidine group (negative control) And after treatment, there was a significant difference in several colonies (p <0.05). A comparison of the number of Staphylococcus Aureus colonies before and after treatment between different groups, according to Post Hoc analysis was presented in Table 2. This analysis showed that in the pre-treatment colonies, Methylene Blue with Indocyanine Green and Chlorhexidine There was no significant difference (p> 0.05) in post-treatment colonies (p> 0.05). However, there was no significant difference between Indocyanine Green and Methylene Blue in terms of colony count (p> 0.05). However, these two substances had a significant difference with Chlorhexidine (p <0.05).

Table 1: Central distribution indexes and standard deviation of Staphylococcus Aureus colony count in a different group.

Groups		Sample Number	Average	Standard deviation
Methylene Blue	Before CFU	20	88.2×10^6	4.021×10^6
	After CFU	20	49.2×10^6	4.81×10^6
Indocyanine Green	Before CFU	20	85.05×10^6	5.94×10^6
	After CFU	20	52.65×10^6	4.78×10^6
Chlorhexidine	Before Negative Control CFU	20	87.6×10^6	4.29×10^6
	After CFU	20	13.15×10^6	5.88×10^6

Table 2: Comparison of two groups of Staphylococcus Aureus colony count before and after treatment according to Post Hoc analysis.

The dependent variable			Average difference	Standard deviation	P value
Colony - Before	Methylene Blue	Indocyanine Green	3.15×10^6	1.52×10^6	0.107
		Chlorhexidine	0.6×10^6	1.52×10^6	0.919
	Indocyanine Green	Methylene Blue	-3.15×10^6	1.52×10^6	0.107
		Chlorhexidine	-2.55×10^6	1.52×10^6	0.225
	Chlorhexidine	Methylene Blue	-0.6×10^6	1.52×10^6	0.919
		Indocyanine Green	2.55×10^6	1.52×10^6	0.225
Colony - After	Methylene Blue	Indocyanine Green	-3.45×10^6	1.64×10^6	0.098
		Chlorhexidine	36.05×10^6	1.64×10^6	0.000
	Indocyanine Green	Methylene Blue	3.45×10^6	1.6×10^6	0.098
		Chlorhexidine	39.5×10^6	1.64×10^6	0.000
	Chlorhexidine	Methylene Blue	-36.05×10^6	1.64×10^6	0.000
		Indocyanine Green	-39.5×10^6	1.64×10^6	0.000

4. Discussion

In this laboratory study, the susceptibility of Staphylococcus Aureus to photodynamic therapy was evaluated using Indocyanine Green and Methylene Blue. Light-sensitive materials with diode laser and compared with the effect of Chlorhexidine bactericide. This study showed that all three experimental groups reduced the number of Staphylococcus Aureus colonies, although the effects of Staphylococcus Aureus reduction were significantly more severe than Chlorhexidine. There was no significant difference between Ingocyanine Green and Methylene Blue. However, these two materials were significantly different from Chlorhexidine, and in some Chlorhexidine samples, no bacterial colony was grown. In many studies, photodynamic therapy has been introduced as a method for the elimination of various microorganisms, including Staphylococcus Aureus. It can be used as an alternative to anti-microbial mouthwashes, such as Chlorhexidine. In 2015, Michael and et al. investigated anti-microbial photodynamic therapy on Staphylococcus Aureus using a Phenothiazine and red laser. The results

showed that the use of sensitizers alone reduced the mean CFU (64.8%) and its correlation with laser light, A 84.2% decrease in bacterial colony count,[29] which, is consistent with our research results. Minghsieh and et al. (2014) investigated the inactivation of amino oleic acid-induced by photodynamic therapy on Staphylococcus Aureus and Pseudomonas aeruginosa. This study showed that ALA combined with red LEDs is a fast and inexpensive method for PDI on Staphylococcus Aureus,[30] and these results are consistent with the current research. Renata Tiemi and et al. reviewed the anti-microbial anti-Staphylococcus Aureus anti-microbial phototherapy UVA / Riboflavin effect in 2012. They concluded that the combination of 0.1% riboflavin and 365 nm UVA did not have an antimicrobial effect against Staphylococcus Aureus.[31] The present study is not compatible, probably because the type of sensitizer is different from the two studies. According to studies, no studies have been found to investigate the effect of photodynamic therapy using methylated Blue and Indocyanine Green sensors on the teeth. According to this, our research is worthwhile, more similar to clinical conditions in the oral environment. It should be noted that when its optimal wavelength stimulates a light-sensitive material, it is energized from low-energy mode and the high half-life of the triple-state results in a reaction between the light-sensitive material and the environment and oxygen molecules in the tissue. Oxygen produces singlet and other free radicals, which causes tissue destruction.[13] Methylene Blue is an alkaline light source that passes through a bacterial cell membrane and acts on the bacterial genome, causing bacteria to disappear. It also generates free oxygen in combination with laser radiation, which eliminates bacteria. Indocyanine Green is an anionic light sensitizer that attaches to the cellular membrane of a positively charged bacterium, which causes the membrane to disappear. It also removes the bacterium in combination with laser radiation with photodynamic therapy. Generally, the bacterioidal effect of photodynamic therapy is described by two mechanisms: 1) DNA damage 2) damage to the cytoplasmic membrane of the bacterium, through cytotoxic agents produced by photodynamic therapy, which itself disables the membrane transfer system and plasma membrane enzymes, Peroxidation and so on. As a result, the permeability of the membrane increases.[18] Also, Chlorhexidine has two mechanisms that affect microorganisms:

1) Connection to the membrane structure followed by bacterial osmosis damping and increased cell permeability and leakage of intracellular ions such as potassium to the outside of the damaged membrane.

2) The formation of intracellular coagulation responsible for the bactericidal activity and dependent on Chlorhexidine concentration.[25]

The mechanisms mentioned above justify the results of this study. Selection of Staphylococcus Aureus in this research and some other studies was because contamination of microorganisms, including Staphylococcus Aureus, in oral saliva, is common in oral tissues. Also, Chlorhexidine was selected to compare the efficacy of the treatment due to the effects of proven bactericide and its prevalence in reducing oral microbial load. To reduce oral bacteria in high-risk individuals with a high cariousness, Chlorhexidine is administered once a day for two weeks, which, for prolonged use, causes unpleasant side effects such as changes in taste buds and taste in the mouth, and changes in the color of the tooth and restorations.[25] Therefore, it is essential to achieve the main factor with an appropriate efficacy that does not have these complications against Staphylococcus Aureus. For this purpose, photodynamic therapy with methionine-sensitive light-sensitive agents Methylene Blue and Indocyanine Green were evaluated with a diode laser.

The weaknesses of this research are to investigate laboratory conditions and find significant differences between clinical and laboratory conditions. Also, this study was performed on premolar teeth and may not apply to all teeth. Altogether, it seems that diode laser radiation, along with light-sensitive Indocyanine Green and Methylene Blue, has a positive effect on the colony reduction of Staphylococcus Aureus. However, further studies are needed to achieve the definitive results of these treatments' effects in conditions Clinical manifestation. On the other hand, this treatment should be done at the office by the dentist, and compared to Chlorhexidine, despite its complications, it is not costly for long-term use in the patient, and it costs more for which may be accepted by the patient. Lower than Chlorhexidine.

5. Conclusion

The results of this study showed that photodynamic therapy using two Methylene Blue and Indocyanine Green photosensitizer and Chlorhexidine mouthwash led to a reduction in the number of Staphylococcus Aureus colonies. However, the effects of the reduction of Staphylococcus Aureus were significantly more severe than Chlorhexidine.

Conflict of Interest

The authors declared that there is no conflict of interest.

Acknowledgments

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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