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The Application of Antimicrobial Photodynamic Therapy on *Pseudomonas aeuroginosa* and *Enterococcus fecalis* using Heperecin and Methylene Blue Photosensitizers

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ABSTRACT

The spread of multi-resistant bacterial strains is a fundamental threat to the public health and increases mortality rates and health care costs .Given that the main reason for resistance in bacteria is over use and misuse of antibiotic compounds. the photodynamic therapy has been introduced as new way to deal with resistant infections. In this empirical in vitro study, the strain of Enterococcus faecalis and the strain of Pseudomonas aeruginosa were prepared as standard strains. Two photosynthesizers of Methylene blue and hypericin as well as solutions of Enterococcus faecalis, and Pseudomonas aeruginosa were tested in 96-well plates. The irradiation process was conducted in the sterilized condition in the darkness as follows:First, they were irradiated for 30, 60 and 120 seconds using laser diodes with a wavelength of 630 nm. None of the colonies of bacteries grew in the presence of hypericin with and without laser irradiation and after 48 hours of incubation. The number of the colonies counted from Enterococcus bacteria in the presence of methylene blue and the radiation rate of both 2.5 and 5 mm Watt lasers were zero after 48 hours of incubation. However, the number of the colonies counted from the Enterococcus faecalis was 36 CFU/mL in the case of exposure to methylene blue with the concentration of 25 µg/mL and without laser irradiation. The results showed that hypericin with and without laser therapy property has a bactericidal property against both Enterococcus faecalis and Pseudomonas aeruginosa.. Moreover, the use of methylene blue at a concentration of 25 µg/mL in the presence of 5 mm-watt laser, has a bactericidal property against Enterococcus faecalis, and reduces the number of Pseudomonas aeruginosa bacteria.

Key word: Heperecin, Methylene blue, Photodynamic therapy, Photosensitizers.

INTRODUCTION

Today, the spread of multi-resistant bacterial strains is a fundamental threat to the public health and increases mortality rates and health care costs[1,2].Given that the main reason for resistance in bacteria is overuse and misuse of antibiotic compounds, efforts are still underway to find an alternative way to deal with bacterial infections[1, 3] and the photodynamic therapy has been introduced as new way to deal with resistant infections[1-3].In this therapy method, sensitive photo-nodes (photosensitizer) are activated under light irradiation and oxygen free radicals are produced[4]. The reactive oxygen radicals cause oxidation of lipids and proteins in the cytoplasmic and nucleic acid membrane and the damages lead to the death of micro-organisms[3]. Since free radicals act totally nonspecificand deactivate various locations in the cell, so far, bacteria showed no resistance against

this method[5, 6].Methylene blue is a chemical dye, which belongs to phenothiazinium compounds and is used in the clinical antimicrobial therapy[7].Methylene blue is considered as important antimicrobial light activator due to its less toxicity levels on human cells as well as its high ability to produce reactive oxygen[7,8].The positive charge of the compound at physiological pH enables the dye to be located in the membrane of gram-negative and gram-positive bacteria[9].Hypericin is a naturally-occurring polycyclic quinine and is obtained from hypericumperforatum L., which is a medicinal plant used in traditional medicine of Iran[3].The substance is a photosynthesizer[10], the anti-bacterial and anti-fangal properties of which have been seen in previous studies[3, 11] .In a study in 2011, Pereira et al. showed that photodynamic therapy, using the photosensitive effects of methylene blue, is a useful method for inhibiting the growth and elimination of oral biofilms, particularly *Candida albicans* and *S. aureus* and *Streptococcus mutans*[12]. Also, in a study in 2012, Kashef et al. showed that hypericin causes photodynamic inactivation of *Enterococcus faecalis, Staphylococcus aureus* and *Escherichia coli*[13]. Considering the outbreak and spread of bacterial strains resistant to antibiotics and their role in infections, sometimes uncontrollable, the effect of PDT (photodynamic therapy) on the bacteria,including Pseudomonas aeruginosa and Enterococcus faecalis was evaluated and comparedin this study. Moreover, considering the type of the photosynthesizer is effective on the laser penetration and thus, the result of the PDT effect, Pseudomonas aeruginosa and Enterococcus faecalis were separately used from two photosynthesizers (H. perforatum and methylene blue) and their effects were compared with each other in each of the cases.

MATERIALS AND METHODS

In this empirical in vitro study, the strain of *Enterococcus faecalis* (MMH504) and the strain of *Pseudomonas aeruginosa* (ATCC27853) were prepared as standard strains from Scientific and Industrial Research of Iran.The bacteria were separately cultured in Brain Heart Infusion (BHI) medium. All media were maintained at the incubator at 37 ° C and under aerobic conditions for 48 hours. New colonies of Enterococcus faecalis, and *Pseudomonas aeruginosa* were suspended from Müller-Hinton agar plates (MH) in BHI medium and the bacterial density was set at McFarland 0.5 opacity. Moreover, the liquid media containing the intended bacteria were maintained at the incubator at 37 ° C under aerobic conditions for 24 hours. Then logarithmic phase organisms were centrifuged at 3000g for 15 minutes and the liquid floating on the surface was removed. Then, the residue was washed 2 or 3 times using sterilized sodium phosphate buffer.The sterilized buffer was added and the cell suspension was prepared (almost 108CFU (Colony Forming Units)/ ml[14].Methylene blue (MB) was purchased from Merk CO.To obtain the concentration of 25 µg / mL, the methylene blue powder was dissolved in the distilled water. Hypericumperforatum L. extract, which was purchased from Poursina CO, contained 0.1 mg / mL of hypericin . Two photosynthesizers of Methylene blue (25 and 50 µg / mL) and hypericin(100,50,20 and10 µg / mL) as well as solutions of Enterococcus faecalis, and Pseudomonas aeruginosa were tested in 96-well plates. In the first experiment, 100 µg / mL of hypericin and 25 µg / mL of methylene blue as well as solutions of Enterococcus faecalis, and Pseudomonas aeruginosa were separated from each other in 96-well plates (Table 1).

In the second experiment, the concentrations of 100 and 10 µg / mL of hypericin and 25 µg / mL of methylene blue and solutions of *Enterococcus faecalis*, and *Pseudomonas aeruginosa* were separated in 96-well plates. (Table 2)

In the third experiment, concentrations of 50 and 20 µg / mL of hypericin and 50 µg / mL of methylene blue and solutions of Enterococcus faecalis, and Pseudomonas aeruginosa were separated in 96-well plates . (Table 3)

Sterile phosphate-buffered saline (PBS) was added for unification of the liquid surface in the wells in all first, second and third experiments in laser and control groups.

In each well, 50 µL of the suspension of each bacterium was added to 50 µL of photosynthesizers. Before irradiation, samples

were kept in the dark for 5 minutes. The irradiation process was conducted in the sterilized condition in the darkness as follows:

First, they were irradiated for 30, 60 and 120 seconds using laser diodes with a wavelength of 630 nm and a power of 2.5 mW 5(Brand: PASCO Scientific and model:LA-23891 V Made in China) with stability of more than 95 %. The light source was fixed vertically to prevent spread of light in the adjacent wells. The distance between adjacent samples was equal to the width of two wells ,which were covered using black coating.

After these steps, plates were incubated in the first experiment; however, the incubation wan not performed in the second and third experiments and samples were diluted in the PBS. In order to assess the bacterial viability, 50 µL of each diluted sample was cultured in the Müller-Hinton agar and was incubated for 24 hours at relative space of CO 25%. After incubation, to count the total microbial colonies, the intended microbe reached the specific volume and then it was cultured on the surface of the medium. Later, the created colonies were counted after 24 hours and the final number of remaining microbes was assessed in CFU / mL (14, 15) for the prepared dilution.

These experiments were performed in triplicate and data obtained from the study were analyzed using descriptive statistics.

Findings

None of the colonies of *Enterococcus faecalis*, and *Pseudomonas aeruginosa* grew in the presence of hypericin with concentration of 100µg / mL with and without laser irradiation and after 48 hours of incubation. The number of the colonies counted from Enterococcus bacteria in the presence of methylene blue(25µg / mL) and the radiation rate of both 2.5 and 5 mm Watt lasers were zero after 48 hours of incubation.However, the number of the colonies counted from the Enterococcus faecalis was 36 CFU/mL in the case of exposure to methylene blue with the concentration of 25µg / mL and without laser irradiation. The experiments were repeated three times and the results were exactly the same each time(Table 4).

Table 1: Grouping of different environments in the first experiment

1	2	3	4	5	6
($L^+MB^+H^-$)	($L^+ H^+ MB^-$)	($L^+ H^- MB^-$)	($L^-MB^+H^-$)	($L^- H^+ MB^-$)	($L^- H^- MB^-$)

L=laser MB=Methylene blue H= hypericumperforatum L.

Table 2: Grouping of different environments in the second test

1	2	3	4	5	6	7	8
($L^+MB^+H^-$)	($L^+ H^+ MB^-$)	($L^+ H^- MB^-$)	($L^-MB^+H^-$)	($L^- H^+ MB^-$)	($L^- H^- M^-$)	($L^+ H^+ MB^-$)	($L^- H^+ MB^-$)

L=laser MB=Methylene blue H= hypericumperforatum L.

Table 3: Grouping of different environments in the third test

1	2	3	4	5	6	7	8
($L^+MB^+H^-$)	($L^+ H^+ MB^-$)	($L^+ H^+ MB^-$)	($L^+MB^-H^-$)	($L^- H^-MB^+$)	($L^- H^+ MB^-$)	($L^- H^+ MB^-$)	($L^- H^- MB^-$)

L=laser MB=Methylene blue H= hypericumperforatum L.

Table 4: Number of colonies of *Pseudomonas aeruginosa*, *Enterococcus faecalis* in the presence of hypericin and methylene blue with and without exposure to laser

Environment exposure	Number of colonies (CFU / mL) in the non-laser environment		Number of colonies (CFU / mL) in case of the exposure to 2.5mm Watts laser		Number of colonies (CFU / mL) in case of the exposure to 5 mm Watts laser	
Photosynthesizer	**PA**	**EF**	**PA**	**EF**	**PA**	**EF**
Hypericin (100µg/mL)	0	0	0	0	0	0
methylene blue(25µg/mL)	80,000	36	50,000	0	20,000	0

PA: *pseudomonas aeruginosa* EF: *enterococcus faecalis*

Table 5: Number of colonies of *Pseudomonas aeruginosa*, *Enterococcus faecalis* in the presence of different concentrations of hypericin with and without exposure to laser

Hypericin concentrations	Number of colonies (CFU / mL) in the non-laser environment		Number of colonies (CFU / mL) in case of the exposure to 2.5mm Watts laser		Number of colonies (CFU / mL) in case of the exposure to 5 mm Watts laser	
	PA	**EF**	**PA**	**EF**	**PA**	**EF**
100µg/mL	0	0	0	0	0	0
50µg/mL	80,000	100,000	50,000	100,000	200	5,000
20 µg/mL	100,000	100,000	80,000	100,000	20,000	50,000
10 µg/mL	100,000	100,000	100,000	100,000	80,000	80,000

PA: *pseudomonas aeruginosa* EF: *enterococcus faecalis*

Table 6: Number of colonies of *Pseudomonas aeruginosa*, *Enterococcus faecalis* in the presence of different concentrations of methylene blue with or without exposure to laser

Methylene blue concentrations	Number of colonies (CFU / mL) in the non-laser environment		Number of colonies (CFU / mL) in case of the exposure to 2.5mm Watts laser		Number of colonies (CFU / mL) in case of the exposure to 5 mm Watts laser	
	PA	**EF**	**PA**	**EF**	**PA**	**EF**
25 µg/mL	80,000	36	50,000	0	20,000	0,0
50µg/mL	100,000	100,000	100,000	100,000	80,000	0,0

PA: *pseudomonas aeruginosa* EF: *enterococcus faecalis*

The above experiment was repeated once with one minute irradiation time and once more with two minutes irradiation time and the results were exactly similar to the 30-second irradiation. In the next step, microorganisms were irradiated at 30 seconds and were added into the culture medium without incubation. Then, the colonies were counted. Also at this stage, to determine the optimum concentration, hypericin(100, 50, 20 and 10µg/mL)and methylene blue(50 and 25 µg/mL)were evaluated separately.

The number of colonies was zero when hypericin at concentration rate of 100 µg/mL was used, both in the presence and in the absence of laser. With a decrease in the concentration of hypericin to less than 100µg / mL, microorganisms grew in the culture medium and the number of colonies increases. The number of colonies of Pseudomonas aeruginosa was 80000 CFU/mL when it was exposed to hypericin with concentration rate of 50 µg / mL(Table 5).Enterococcus faecalis was completely eliminated when it was exposed to hypericin with the concentration of 100 µg/mL. Moreover, the number of colonies was zero in the presence of laser and non-laser exposure. Again,microorganisms grew in the culture medium and the number of colonies increased with a decrease in the hypericin to less than 100µg / mL.

The number of colonies of Enterococcus faecalis was 100000 CFU/mL when it was exposed to hypericin with concentration rate of 50 µg / mL.These experiments were repeated three times and each time the results were exactly the same. (Table 5)

The above experiment was repeated once with one minute irradiation time and once more with two minutes irradiation time and the results were exactly similar to the 30-second irradiation. With an increase in the concentration of methylene blue to the amount of more than 25µg / mL, the bacterial growth rate was increased in the culture medium.The number of colonies grown from P. aeruginosa was 80000 CFU / mL in the presence of methylene blue at a concentration of 25µg / mL.When the culture medium of the Pseudomonas aeruginosa was irradiated with 2.5 and 5 mm-watt laser, the number of counted colonies was reduced by the values of 50000 and 20000 CFU / mL, respectively.

The number of colonies grown from Enterococcus faecalis, which were exposed to 25µg / mL methylene blue, was 36 CFU / mL,0 and 0 respectively in non-laser environment, radiation with 2.5 and 5 mm-watt lasers. These experiments were repeated three times and each time the results were exactly the same (Table 6)

The above experiment was repeated once with one minute irradiation time and once more with two minutes irradiation time and the results were exactly similar to the 30-second irradiation.

DISCUSSION

Pseudomonas aeruginosa and enterococci faecalis have the ability to create antibiotic resistance and efforts are still underway to find a non-antibiotic treatment[16]. This study was performed to compare the effects of photodynamic therapy on two microorganisms of Enterococcus faecalis, and Pseudomonas aeruginosain the presence of methylene blue and hypericin. The results showed that the use of the hypericin(100µg / mL) with and without laser therapy, has the bactericidal property against both Enterococcus faecalis and Pseudomonas aeruginosa. In a study conducted in 2011, Ali et al. showed that hypericumperforatum L. extract has antibacterial property against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[17]. In another study conducted in 2007, Milosevic et al, showed that hypericumperforatum L. extract has antibacterial property against gram-positive and gram-negative bacteria, especially Pseudomonas family. Cervenka et al in 2006[18] and Brantner and colleagues in 2006, investigated the anti-bacterial property of hypericin and respectively concluded that it has the anti-bacterial property against Arcobacter and *Staphylococcus aureus* strains[19], which is consistent with the results of this study. In the present study, comparison of the concentrations of hypericin(100, 50, 20 and 10µg/mL) showed that reducing the hypericin concentrations to less than 100µg / mL, its anti-bacterial propertyand growth of micro-organisms in the culture medium will be reduced. In general, the use of hypericin(100µg /

mL)with and without laser therapy, and hypericin (50µg / mL) in the presence of 5 mm-watt laser will lead to a reduction in its bactericidal property and the number of the bacteria. In a study conducted in 2012, Kashef et al. investigated the effect of hypericin (of 0.1, 0.3, 0.6 and 1 µg / mL) and light irradiation time of(3, 5 and 10 minutes) on photodynamic inactivation of microorganisms and concluded that hypericin along with a photo dose of 48 J/cm^2 reduced the growth of microorganisms of *Enterococcus faecalis, Staphylococcus aureus* and *Escherichia coli*. It also has a bactericidal property, but Pseudomonas aeruginosa had relative resistance against other micro-organisms[13]. However, in this study, *Pseudomonas aeruginosa*, compared with *Enterococcus faecalis*, was more sensitive to hypericin with and without laser therapy. The reason for this difference may be related to difference in the concentrations of hypericin in two studies or differences in the type of light source used.In another study, Rezusta and colleagues in 2011 concluded that the growth of different species of Candida fungus was significantly reduced under the influence of hypericin concentrations of 0. 625, 1.25, 2.5 and 40 µM and LED lamp emitting (18 mJ square cm). Furthermore, the antifungal effect was increased with an increase in concentrations of hypericin or light doses[20].

Luthi et al in another study in 2009 on the tooth decay-causing bacteria, showed that streptococcus sobrinus was eliminated following 15 minutes of incubation with a concentration of 2.5 µg/ml of hypericin and illumination time of 120 seconds. Moreover, they showed that a total of 99.9 percent of Streptococcus mutans bacterium was eliminated following 30 minutes incubation with a concentration of 10 µg/ml of hypericin along with illumination frequency of 2 times, each for 120 seconds[21].Yow and colleagues, in a study in 2012, showed that the simultaneous application of hypericin and light irradiation cause adramatic reduction and significant changes in gram positive methicillin-sensitive and resistant *S. aureus* bacteria, but it had no effect on slowing the growth of the *Escherichia coli*, gram-negative bacterium[22].

In the present study, the application of 25 µg/ml methylene blue in the presence of 5 mm-watt laser has a bactericidal property and also it reduces the number of the enterococcus faecalis in the presence of the 2.5 mm/watt laser. However, the use of methylene blue at a concentration of 25 µg/ mlwhether with 5 mm-watt laser or 2.5-watt mm laser, reduces the number of Pseudomonas aeruginosabacteria. Furthermore, with an increase in the concentration of methylene blue to more than 25µg / mL, the bacterial growth was increased in the culture medium. So, it can be concluded that the optimum concentrations of methylene blue is to 25µg / mL.The enterococcus faecalis, compared with Pseudomonas aeruginosa, showed more sensitivity to methylene blue with or without the laser therapy.Fontana et al. in 2009, measured the antibacterial effect of photodynamic therapy by methylene blue and concluded that a total og 63% of bacteria found in dental plaque suspension prepared from samples by photodynamic therapy were destroyed. But the microbial biofilms taken from the same plaque sample showed less sensitivity to the photodynamic therapy and only 32 % of bacteria were killed[15].

In another study, Street and colleagues in 2009 evaluated the effect photodynamic therapy on the P. aeruginosa in the presence of methylene blue. The results of this study showed that planktonic P. aeruginosa was completely eliminated in the presence of methylene blue and a laser with 15 J/ cm^2. In addition, a total of 99.9 percent of the 24 h biofilm viability and 48 h biofilm viability of the bacteria was eliminated in the presence of methylene blue and a laser with energy of 13.2 J/ cm^2 .With a two-fold increase in the exposure to the laser radiation, a greater reduction was observed in the amount of colonies counted from these bacteria[16]. In a study in2009, Araújo and colleagues showed that a concentration of 25µg/mL of methylene blue led to the 73% inhibition of the growth of Streptococcus mutans bacteria in the presence of red light[23]. However, Lozano and colleagues who studied the decay-causing Streptococcus mutans and sanguinis bacteria in 2015 showed that the a total of 99.99 percent of the bactria were eliminated in the presence of the methylene blue at a concentration of 2.5 µg/mL while being exposed to the white light with energy of 37 J/cm^2 and the incubation duration of 60 seconds. In that study, with increasing the incubation time, more anti-bacterial property was

observed. Also in that study, and in similar circumstances but with higher concentrations of methylene blue (80-160 µg / mL), the Candida albicans fungus was eliminated (6). After comparing the results obtained in this study with those from other studies, it can be concluded that the photodynamic efficacy of each photosynthesizer is different than the type of microorganism. Moreover, it must be noted that combining a variety of photosynthesizers with different light sources can be effective in the treatment of infections caused by a combination of microorganisms or microorganisms resistant to common antibiotic treatments.

CONCLUSION

The results showed that hypericin at a concentration of 100µg / mL with and without laser therapy property has a bactericidal property against both Enterococcus faecalis and Pseudomonas aeruginosa. The concentration of 50 µg / mL of hypericin also reduced the number of the bacteria in the presence of the 5 mm watt laser. Moreover, the use of methylene blue at a concentration of 25 µg / mL, in the presence of 5 mm-watt laser, has a bactericidal property against Enterococcus faecalis, and reduces the number of Pseudomonas aeruginosa bacteria.

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