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ORIGINAL ARTICLE

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Antimicrobial effect of methylene blue formulations with oxygen carrier at different pHs: preliminary study

Efeito antimicrobiano de formulações de azul de metileno com carreador de oxigênio em diferentes pHs: um estudo preliminar.

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ABSTRACT

Objective: Evaluate methylene blue (MB) formulations containing oxygen carrier at different pHs in antimicrobial photodynamic therapy (aPDT). **Material and Methods:** Biofilms of *Pseudomonas aeruginosa* PA01 formed over acrylics specimens during five days were treated with aPDT using different formulations: MB/pH 7.4; MB/pH 5.6; MB/carrier pH 7.4; MB/carrier pH 5.6. Biofilms not exposed to treatment were used as a control. Blind examiner for the experimental groups performed the counting of colonies per ml suspension (CFU/ml). Two-way ANOVA was used to determine the effect of factors solvent (carrier vs water) and pH (7.4 vs 5.6). One-way ANOVA and post-hoc Tukey's test was used to evaluate differences among the five groups (control; MB/carrier pH 7.4; MB pH 7.4; MB/carrier pH 5.6; MB pH 5.6). The Statistics 8.0 software was used ($P<0.05$). **Results:** All of photodynamic therapy groups showed significant reduction in *P. aeruginosa* compared to the control group. The solvent factor was not significant ($P=0.18$), while the pH factor presented statistical significance ($P=0.01$). When the carrier was used, MB formulation at pH 7.4 presented a statistically greater reduction of *P. aeruginosa* than the formulation with pH 5.6. **Conclusion:** The PDT using methylene blue formulations with oxygen carrier demonstrated potential for the treatment of localized infections by *P. aeruginosa.* MB formulations with oxygen carrier and pH 7.4 resulted in higher antimicrobial effect and should be considered for future studies with multispecies biofilms.

KEYWORDS

Antimicrobial photodynamic therapy; biofilm; laser; Pseudomonas aeruginosa.

RESUMO

Objetivo: Avaliar formulações de azul de metileno (AM) contendo carreador de oxigênio a diferentes pHs na terapia fotodinâmica antimicrobiana (TFDa). **Material e Métodos:** Biofilmes de Pseudomonas aeruginosa PA01 formados sobre espécimes acrílicos durante cinco dias foram tratados com TFDa utilizando diferentes formulações: AM / pH 7,4; AM / pH 5,6; AM / carreadir pH 7,4; AM / carreador pH 5,6. Biofilmes não expostos ao tratamento foram utilizados como controle. Um examinador cego aos grupos experimentais realizou a contagem de colônias por ml de suspensão (UFC / ml). O teste Anova dois fatores foi utilizado para determinar o efeito dos fatores solvente (carreador vs água) e pH (7,4 vs 5,6). Anova um fator e teste *post-hoc* de Tukey foram utilizados para avaliar as diferenças entre os cinco grupos (controle; AM / carreador pH 7,4; AM pH 7,4; AM / carreador pH 5,6; AM pH 5,6). O software *Statistics* 8.0 foi utilizado ($P<0,05$). **Resultados:** Todos os grupos da terapia fotodinâmica mostraram uma redução significativa na *P. aeruginosa* em comparação ao grupo controle. O fator solvente não foi significante ($P = 0,18$), enquanto o fator pH apresentou significância estatística ($P = 0,01$). Quando o carreador foi utilizado, a formulação AM a pH 7,4 apresentou uma redução estatisticamente maior de *P. aeruginosa* do que a formulação com pH 5,6. **Conclusão:** A TFDa utilizando formulações de AM com carreador de oxigênio mostrou potencial para o tratamento de infecções localizadas por *P. aeruginosa.* As formulações de AM com carreador de oxigênio e pH 7,4 resultaram em maior efeito antimicrobiano e devem ser consideradas para futuros estudos com biofilmes multiespécie.

PALAVRAS-CHAVE

Terapia fotodinâmica antimicrobiana; Biofilme; Laser; *Pseudomonas aeruginosa.*

INTRODUCTION

Antimicrobial photodynamic therapy (aPDT) has been proposed as an adjuvant to antibiotic treatment [1] with low probability of causing bacterial resistance [2], being effective against strains resistant to antibiotics [3]. Attempts to optimize the effect of aPDT such as news photosensitizer (Ps), changes in the solvent of formulations [4,5], addition of oxygen carriers [4], nanoplatforms for the application of Ps [6,7], inhibitors of efflux pump [8,9], antibodies conjugated to Ps [10], have been investigated.

The singlet oxygen, due to its strong oxidative activity [11], has been considered the main responsible for the antimicrobial effect of aPDT [4]. In oral biofilms, especially in those located in subgingival environments, there are areas with different oxygen gradients and under anaerobiosis conditions. Therefore, the combination of Ps and light may not be sufficient for the effective eradication of biofilm [12]. Strategies to improve oxygenation in systems subject to photodynamic therapy have been studied [13]. The use of oxygen carriers could increase the availability of oxygen and facilitate the propagation of light during the irradiation, increasing the photochemical oxidation potential, facilitating the disruption of the biofilm matrix and the eradication of bacteria [12,14]. Perfluorocarbons (PFCs), such as perfluorideecalene, is a petroleum derivative synthesized by the substitution of hydrogen atoms for fluorine atoms in hydrocarbon molecules. The solubility of oxygen in PFCs is 10 to 20 times greater than in water, and their characteristics significantly increase the rate of oxygen transfer from the gas phase to microorganisms [15,16]. Kamuhabawa et al., in 2006, observed that the addition of perfluorecarene resulted in enhanced effect of photodynamic therapy with hypericin inducing increased apoptosis of tumor cell carcinoma [17].

The pH-dependent behavior of some Ps has been investigated [18,19]. The amount of hydroxyl radicals (OH) present in the environment increases according to the pH solution. The more basic the pH, more hydroxyl radicals (OH). These radicals react with biomolecules or combine with each other and form hydrogen peroxide, which has cytotoxic effects [20]. Studies have shown that pH can alter the fluorescence spectrum and chlorine absorbance. It may still alter molecular characteristics and the interaction of Ps with tumor cells during photodynamic therapy [21-23]. There is evidence that the production of singlet oxygen can be potentiated according to the pH [24]. Photosensitive phthalocyanines present greater potential to produce reactive oxygen species (ROS) and singlet oxygen in acid environment [19].

There is no evidence demonstrating the effect of pH or the addition of oxygen carrier on the antimicrobial effect of photodynamic therapy when methylene blue (MB) is used as Ps. Moreover, we used *Pseudomonas aeruginosa* biofilm. In biofilms, P. aeruginosa resistance to antibiotics can be 10 to 1,000 times greater than that in planktonic culture [25] Therefore, the P. aeruginosa biofilm consists in an important model for evaluating the antimicrobial effect of the photodynamic therapy.

The objectives of this pilot study were: (1) to compare the antimicrobial effect of aPDT among formulations containing MB in aqueous solution with an experimental formulation containing MB and an oxygen carrier substance; and (2) to evaluate the pH effect of MB formulations on antimicrobial effect on *P. aeruginosa* biofilms. Our hypothesis considered that the formulation containing the oxygen carrier would result in the highest antimicrobial effect.

MATERIAL AND METHODS

The Ps used was methylene blue (Sigma Aldrich®, São Paulo, SP, Brazil). Solutions were prepared using ultra-pure water (Milli-Q). To prepare the buffered solutions the following materials were used: Tris (hydroxymethyl) aminomethane (Sigma Aldrich®) and hydrochloric acid (Vetec®, Rio de Janeiro, RJ,

Brazil) for pH 7.4; and acetic acid (Vetec®) and sodium acetate (Proquímios®, Rio de Janeiro, RJ, Brazil) for pH 5.6. Perfluordecalene (Acros Organics®, New Jersey, USA) was used as an oxygen carrier, and triton-X100 (Sigma Aldrich® São Paulo, SP, Brazil) was used as a surfactant in the preparation of the emulsions.

The pH of the solutions was adjusted using the DM-20 pH meter (Digimed®, São Paulo, SP, Brazil), and solutions of hydrochloric acid and sodium hydroxide (Vetec®) were used to achieve the expected pH.

The light source used was an Indium-Gallium-Aluminum-Phosphorus laser (InGaAlP, Thera Lase - DMC, São Carlos, SP, Brazil) with a wavelength of 660nm, fiber tip diameter of 0.02827cm^2, and continuous emission mode. The formulations tested are shown in Table 1.

Table 1 - Formulations of methylene blue (MB) at different pHs submitted to the irradiation.

Formulations	Formultion compositions
MB pH 7.4	MB (0.01%) diluted in buffer (Tris-HCl)
MB/carrier* pH 7.4	MB (0.01%) diluted in a perfluorodecalene: buffer (Tris-HCl): triton-X100 ratio 60: 35: 5,
MB pH 5.6	MB (0.01%) diluted in buffer (sodium acetate/ acetic acid);
MB/carrier* pH 5.6	MB (0.01%) diluted in a perfluorecarene emulsion: buffer (sodium acetate/ acetic acid): triton -X100 60: 35: 5

*oxygen carrier

Antimicrobial effect of MB in different biofilm formulations *in vitro*

Biofilms of standard laboratory strains of P. aeruginosa PA01 (gram-negative) were formed in vitro on acrylic disks with an 8mm diameter. A standardized suspension containing 10^7 cells/ml of microorganism was obtained with a spectrophotometer (Instrutherm, UV-1000A, São Paulo, SP, Brazil). Optical density parameter of 1 (OD_{600nm}) was used.

Sterile acrylic specimens (measuring 8 mm in diameter, 1 mm in height) were placed in a 24-well plate (Costar Corning, New York,NY, USA). Two milliliters (2 mL) of Brain Heart Infusion broth (BHI, Himedia Laboratories PVT. Ltd., Mumbai, India) plus 5% sucrose was pipetted into each well. BHI-immersed specimens were inoculated with 100 μL of standardized microbial suspension (10^7 cells/ml) and incubated in an orbital shaker (Novatecnica, Model NT712, Piracicaba, SP, Brazil) under 75 rpm and 37°C for 5 days. The broth was refreshed every 24 hours. After the incubation period, the specimens were washed with 2 ml of buffered saline solution with phosphate (PBS), to remove the cells.

Photodynamic Inactivation of Biofilms

After washing with saline solution, the specimens were transferred to new 24-well plates and immersed in 1ml Ps (250μM) in the different formulations. The Ps solution was maintained in contact with the biofilm for 5min without light application (incubation period) and afterwards, the biofilms were irradiated with low power laser with 660nm wavelength, in continuous emission mode, usable power of 30mW, energy of 20J and energy density of 40J/cm^2, for 10min.

Afterwards, each specimen containing biofilm was placed in falcon tube containing 10ml of saline solution and placed under vortexing (VELP Scientifica, 12 dc) with power of 15W for 30s. Decimal dilutions were performed from the homogenized solution (10^{-1}). One-hundred microliters (100μl) aliquots of each dilution were seeded in Petri dishes containing MacConkey agar (Himedia Laboratories PVT, Ltd., Mumbai, India).

Plates were incubated at 37°C for 48h. After that period, a blind examiner for the experimental groups counted the colonies formed on the plates and the colony forming unit (CFU) calculation per mL of suspension (CFU/ml).

Five specimens per formulation were used. The experiment was carried out in triplicate. The biofilm formed on the specimens and not exposed to treatment was used as control.

Statistical analysis

The data CFU/ml (Log) were presented on average, standard deviation and 95% confidence interval. Two-way ANOVA was used to evaluate the effect of pH factor (7.4 versus 5.6) and solvent factor (aqueous versus carrier solution). One-way ANOVA and post hoc Tukey's were used to assess the differences among the five groups (control; MB/carrier 7.4; MB 7.4; MB/carrier 5.6; MB 5.6). Significance level of 5% was used for all evaluations. Statistics 8.0 (StatSoft. Inc., Tulsa, OK, United States) program was used for all analyzes.

RESULTS

The solvent factor (water versus carrier) was not statistically significant (P=0.18), while the pH factor (7.4 versus 5.6) presented statistical significance (P=0.01). The interaction of factors was not significant (P=0.62).

The data CFU/ml (Log) in the experimental groups are presented in Table 2. All groups of aPDT presented statistically significant reduction of *P. aeruginosa* in comparison to the absence of treatment. No difference was found between groups with the same pH. When water was used as solvent, no difference was observed between pH 5.6 and 7.4. When the oxygen carrier was used, the MB formulation at pH 7.4 exhibited statistically significant reduction of *P. aeruginosa* compared to the formulation at pH 5.6.

Table 2 - Mean CFU/mL ± standard deviation for the biofilms of *P. aeruginosa* in the control group (no treatment) and in the other antimicrobial photodynamic therapy groups.

	Experimental groups	
	pH 7.4	pH 5.6
Control/no treatment	6.88 ± 0.14 (6.71-7.06) a	
MB/carrier	3.54 ± 0.78 (2.56-4.52) b	4.86 ± 0.50 (4.22-5.49) c
MB/water	4.12 ± 0.70 (3.24-5.00) bc	5.13 ± 0.68 (4.28-5.97) c

Anova one-way, post hoc Tukey
Different letters: represent statistically significant difference among the groups (P<0.05).

DISCUSSION

The results demonstrated that the inclusion of the oxygen carrier in the MB formulation did not increase antimicrobial effect in relation to the commonly used formulation containing MB in aqueous solution, which disproved our initial hypothesis. We observed that most basic pH had statistically significant effect on the antimicrobial photodynamic effect. Formulations with pH 7.4 had lower mean values of CFU/ml than those with pH 5.6. Furthermore, the formulation containing MB/carrier at pH 7.4 showed statistically higher antimicrobial effect than formulations with pH 5.6.

The most basic pH (7.4) favored the antimicrobial effect. The mechanism(s) that explain this effect need to be elucidated. Previous publications indicate the effect of pH seems to vary according to the characteristics of the Ps used [18,19]. However, there is evidence that for some Ps the basic pH seems to provide greater amount of hydroxyl radicals (OH) in the environment. These radicals react with biomolecules or combine with each other and form hydrogen peroxide, which has cytotoxic effects [18,19].

Theoretical basis for the use of an oxygen carrier would be the possibility of increasing the oxygen supply in the biofilm favoring the production of singlet oxygen via photodynamic activation and, consequently, increasing the antimicrobial effect [12,26]. However, our experiments didn't observe this effect. The use of the carrier may not necessarily have driven more oxygen to the biofilm. Thus, further experiments adding oxygen-carrying substances together with the carrier can be performed.

The aPDT used in the present study, regardless of the formulation or pH used, were effective, since they presented statistically significant reduction in viability *P. aeruginosa* in all groups in comparison to the control group (no treatment). These results confirm previous studies demonstrating that aPDT are effective in several species of microorganisms [27,28]. The concentration of MB used in the present

study was based on a previous study that demonstrated that formulations of 0.01% MB resulted in significantly higher production of singlet oxygen when compared to concentrations of 0.001%, 0.0001% and 0.1% [29]. The choice of biofilms of *P. aeruginosa* was guided by the following factors: (I) being a Gram-negative bacteria, which are naturally more resistant to aPDT when compared to Gram-positive ones [30,31]; due to the outer membrane presenting lipopolysaccharides (LPS) and pore channels that act as a barrier to the photosensitizer penetration; (II) due to its high degree of genomic flexibility, expressing several phenotypes, thus recognized by its intrinsic resistance to antibiotics [32,33], which may be 10 to 1000 times higher than in planktonic culture [34]; (III) by previous evidence demonstrating the need for a higher dose of energy and higher concentration of Ps to alter the viability of *P. aeruginosa* when compared to other microorganisms [30].

In the present study only used untreated biofilm as a control group. The literature has pointed out that MB without photoactivation, or laser without MB, has little or no antimicrobial effect [35], justifying the absence of these comparison groups. However, as limitations of this study, we pointed out that biofilm exposure to buffer solutions and to the oxygen carrier could have been performed to verify if these substances have any antimicrobial effect. Another limited aspect is the reduced number of samples used (n = 5), which compromises the power of the study to identify statistical significance in the tests. However, this is a preliminary study. Moreover, more than statistical significance, we could consider that a difference of approximately 2 log CFU/ml among treatments could be considered microbiologically important in reducing the viability of P. aeruginosa in biofilms [36]. In addition, our findings are in agreement with other studies that used this type of pH [37-39].

The magnitude of the microbial reduction observed in the formulation containing MB/ oxygen carrier at pH 7.4 was similar to that observed with antibiotics on *P. aeruginosa* biofilms [40,41]. This demonstrates that aPDT with these formulations may be a viable alternative and with no risk of adverse reactions or development of microbial resistance.

CONCLUSION

The present study demonstrated that formulations containing MB and oxygen carrier with pH 7.4 resulted in higher antimicrobial effect when compared to pH 5.6 during aPDT on *P. aeruginosa* biofilms. Thus, aPDT with such formulation seems to be promising to act as a supporting agent in the treatment of localized infections. Our preliminary results need be confirmed in models involving multi-species biofilms.

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