Antimicrobial Photodynamic Therapy with Methylene Blue and Urea in *Escherichia Coli* and *Staphylococcus Aureus*

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Abstract. Antimicrobial Photodynamic Therapy (aPDT) is the technique in which a photosensitizing agent (PS) in the presence of oxygen is activated by light of a specific wavelength, resonant to the PS, generating reactive oxygen species through the photodynamic process, promoting the microbial death from oxidative damage. The aim of this study was to evaluate the efficacy of aPDT with methylene blue (MB) and MB with urea (UMB) in bacteria as a proof of concept of the urea-disaggregating action on phenothiazine PS. Sterile dental diamond burs were contaminated with gram negative *Escherichia coli (E. coli*) and gram positive *Staphylococcus aureus (S. aureus*) bacteria (10 8 CFU/mL) and divided into three groups (n = 9): GMB (MB + red laser- RL), GUMB (urea +MB + RL), GC (control-without treatment). The contaminated diamond burs were immersed in tubes with aqueous solution of MB (60 μM) (GMB) or MB diluted in urea (60 μM) (GUMB). After 1 min, irradiation was performed with RL (660 nm, 100 mW, 18 J, 3 min), in contact below and above the tube. The results presented in colony forming units (CFU/ml) showed that the MB and UMB groups showed significantly greater microbial reduction than GC ($p < 0.05$), for all microorganisms. The microbial reduction of the group with urea (GUMB) was not superior to the group without urea (GMB). It can be concluded that aPDT with MB and UMB promoted effective antimicrobial actions, the disaggregation factor that urea has in phenothiazine PS, in this in vitro experiment, was not determinant in microorganisms in suspension.

Keywords: Antimicrobial · Methylene blue · Photodynamic therapy · Urea

1 Introduction

Antimicrobial Photodynamic Therapy (aPDT) is used for oncological treatment and antimicrobial therapy, proving to be an effective method through oxidative processes. aPDT is a photosensitizing agent (PS), it is activated by light of specific wavelength resonant to the PS, triggering the production of singlet oxygen, superoxides and free radicals (reactive oxygen species), which are cytotoxic to target cells [3, 4]. This treatment modality is also called antimicrobial photodynamic chemotherapy, photoactivated disinfection or light activated disinfection.

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Reactive oxygen production is characterized by photochemical oxygen consumption and occurs by inducing two types of reactions. In the type I reaction there is a transfer of electrons or hydrogen, leading to the production of different types of free radicals, superoxides, hydroxyl radicals and hydrogen peroxide, while in the type II reaction there is energy transfer to oxygen by the change in electron spin, leading to the production of singlet or superoxide oxygen, which are highly reactive species [6]. Both lead to cell death by the oxidation of biological molecules such as proteins, nucleic acids and lipids [7]. The action of aPDT extends to bacteria, fungi, yeasts, viruses and protozoa, depending on the SF and light source used, being therefore very useful in combating localized infections [8].

The therapy has different action on gram-positive and gram-negative bacteria, due to structural differences in cell walls. The former are more susceptible to elimination by aPDT. Gram-negative bacteria have a complex outer membrane, with two lipid layers that act as a barrier between the cell and the environment, which makes it difficult to kill them [5].

The success of aPDT depends on the interaction of the light source (laser or LED) with natural or synthetic PS, in the presence of oxygen. The PS must present favorable photo physical, chemical and biological properties. The ability to penetrate bacterial cells is a high absorption coefficient in the region of the light excitation spectrum, ability to transfer energy to species, in addition to having local action and presenting a short time interval between the period of administration and tissue absorption.

Phenothiazine PS are composed of a tricyclic aromatic ring, such as methylene blue (MB) and toluidine blue (TB), they are performed for photodynamic therapy studies [1.8] and are activated by light in the spectrum from 620 to 700 nm (red wavelength [4, 8]. MB and TB have similar chemical and physicochemical characteristics, with hydrophilic nature, low molecular weight and positive charge that facilitates the passage through the bacterial wall [4], inducing damage to the nucleic acids, proteins and lipids [12] The TB has the additional advantage of an affinity for the lipopolysaccharide (LPS) of Gram-negative bacteria and is, in general, more effective than MB.

The addition of urea to aqueous formulations of PS could improve the efficiency of phenothiazine photosensitizers [13] due to its characteristics that lead to PS breakdown as MB tends to aggregate, negatively interfering with the generation of singlet oxygen [14, 15].

Urea weakens hydrophobic bonds, changes the dielectric constant, and increases the surface tension of water, which generally causes a decrease in substrate-substrate interactions, such as those found in ion pairs. Urea stabilizes the solution monomers (and consequently reduces dimer concentration) of MB, allowing aPDT to be more efficient in *Candida albicans* [13].

This study proposes to evaluate in vitro the effectiveness of the decontamination process with MB and TB with urea in dental diamond burs using aPDT with red laser in gram negative bacteria *E. coli* and gram positive bacteria *S. aureus*, being a proof of concept to determine a more refined methodology in new experiments both in vitro and in vivo. The hypothesis of this study is that the addition of urea to methylene blue increases the efficacy of phenothiazine PS.

2 Methods

2.1 Bacterial Samples and Cultivation

Bacterial samples of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) provided by the microbiology laboratory at Universidade Brasil (São Paulo) to conduct the research. For the cultivation of microorganisms, 100 μL of the bacteria solution were incubated in 10 mL of sterile BHI broth, in a test tube and kept in a bacteriological oven (37 °C, 8 h). Tubes with bacteria were standardized with turbidity 7 on the Mc Farland scale, which corresponds to the approximate number of bacteria in the order of 21×10^8.

2.2 Procedures

Conical diamond burs (DB) (Fava, São Paulo, Brazil) for dental use were sterilized and individually immersed in test tubes containing broths with 10^8 CFU/mL of *E. coli* and *S. aureus* in suspension. After a period in a bacteriological oven (37ºC, 16 h) the contaminated DB were divided into three groups (n = 9): GMB (treatment group with MB + red laser − RL), GUMB (MB with urea additive + RL), GC (control group without treatment) (Table 1).

Table 1. Experimental groups

Methylene blue (MB) treatment group	Methylene blue (MB) + Red laser	n = 9
Treatment group with urea-added methylene blue (UMB)	Methylene blue (MB) + urea + Red laser	n = 9
Control Group – no treatment (C)	PS (–) Red laser (–)	n = 9

The contaminated DB were individually immersed in 1.5 ml tubes containing 60 μM aqueous MB solution diluted in MiliQ water (GMB) or 60 μM MB diluted in urea aqueous solution (GUMB) for 1 min (pre-irradiation – TPI). Soon after, irradiation was performed with a red laser (Laser DUO®, MM Optics; Brazil) with a wavelength of 660 nm, power of 100 mW. Irradiation was performed perpendicularly in contact with the tube for 3 min (18 J), 1.5 min above (9 J) and 1.5 min (9 J) below the tube (Fig. 1). Before irradiation, the energy of the Laser equipment was measured.

Afterwards, the diamond burs were removed from the tubes containing the FS and placed in a new tube containing sterile PBS. After centrifugation, the PBS supernatant and the diamond tip were discarded, leaving only the pellet at the end of the tube, in which 100 μL of sterile PBS was added. From this content, 100 μL was taken and placed in the microplate for serial dilution. After serial dilution, 10μL of each dilution were taken to inoculate plates containing BHI culture medium to determine the number of colony forming units (CFU) for each diamond bur. The plates were placed in a bacteriological oven (37 °C, 16 h). This procedure was performed in triplicate and the results obtained were expressed in CFU.

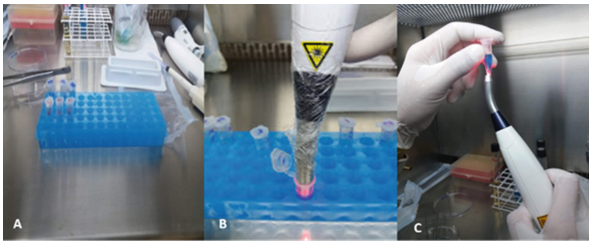

Fig. 1. Illustrative images of the experiment: **A** – Pre-irradiation time; **B** – Irradiation with red laser above the tube containing PS; **C** – laser irradiation under the tube.

3 Results

The samples of microbiological material were collected and submitted to laboratory processing to perform the colony forming units (CFU) count at different dilutions. The experiment was carried out in triplicate and from the original CFU data of each group, calculations of the averages of the different groups were performed.

The statistical analysis of the data (CFU/ml) showed, by the Shapiro-Wilk test, the non-normal distribution of the data in the curve. From this, the Kruskal-Wallis test was performed, in this, as it is a non-parametric test, the original average values (CFU/ml) were transformed into average ranks, and later, the Dunn's Test was used to compare the groups ($p < 0.05$).

As seen in the box-plot chart (Fig. 2). The inferential statistical analysis of the data showed that the MB and UMB groups showed significantly greater microbial reduction ($p < 0.05$) for the *E. coli* compared to the C group. The same can be observed for the *S. aureus*, showing significant greater microbial reduction ($p < 0.05$) in the MB and UMB groups compared to the C group. Such findings, with the limitations of the in vitro study, show the antimicrobial effectiveness of Photodynamic Therapy with MB and UMB in the parameters evaluated in the present study.

No statistically significant differences ($p > 0.05$) were observed between *E. coli* and *S. aureus* bacteria for GC, GMB and GUMB. Such findings show that the study as a proof of concept, did not confirm the disaggregating effectiveness of urea associated with MB in aPDT in vitro.

The action of urea in association with methylene blue on aPDT did not promote effects on different gram-positive and gram-negative bacterial species, and the action should be considered due to differences in the structural and compositional characteristics of these bacteria.

It is interesting to note that there were no statistically significant differences ($p > 0.05$) between the aPDT groups treated with MB or UMB, despite a trend of superiority of UMB for *E. coli* in relation to UMB for *S. aureus*, even being microorganisms with different structural characteristics, being a positive gram and a negative gram.

Finally, the values were converted into results of the microbial survival fraction (CFU/ml) showing the clear difference of remaining microorganisms between the MB and UMB groups and the C group for *E. coli* and *S. aureus* bacteria, becoming evident

the antimicrobial effectiveness of aPDT, and similarity of both techniques with MB and UMB, as confirmed by the statistical analysis (Figs. 3 and 4).

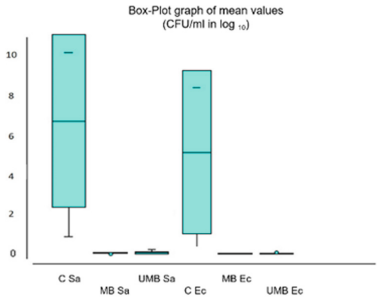

Fig. 2. Box-Plot graph of mean values (±SD) (CFU/ml in log) of groups C *E. coli* (C Ec), MB *E. Coli* (MB Ec), UMB *E. coli* (UMB Ec), C *S. aureus* (C Sa), MB *S. aureus* (MB Sa), UMB *S. aureus* (UMB Sa)

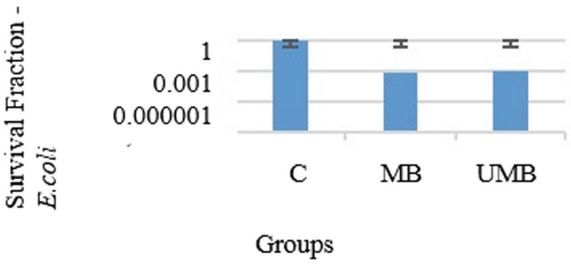

Fig. 3. Graph of the results of the Microbial Survival Fraction (CFU/ml) of groups C, MB and UMB for Gram negative bacteria *E. coli*

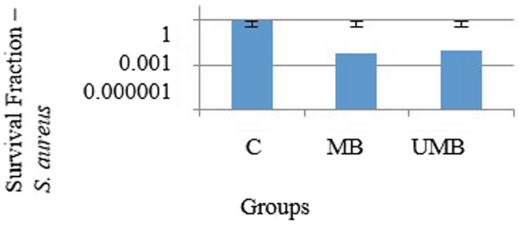

Fig. 4. Graph of the results of the Microbial Survival Fraction (CFU/ml) of groups C, MB and UMB for Gram positive *S. aureus*

4 Discussion

Antimicrobial photodynamic therapy (aPDT) has effective antimicrobial action and applications in different areas of health, and is a therapy based on the association of a PS, a source of light with a specific wavelength and oxygen, generating the production of species through the photodynamic process. Reactive oxygen specimens (ROS)

promoting microbial death by oxidative damage. MB is the main PS used in studies and clinical practice. They are currently looking for new PS or association with chemical compounds, aiming to increase antimicrobial effectiveness [5, 6, 9–11].

This study is intended to be a proof of concept in the use of aPDT with phenothiazine PS added with urea, to determine parameters and define a protocol for use to be followed in other in vitro and in vivo experiments, as MB tends to aggregate, negatively interfering with the generation of singlet oxygen [6, 11]. The MB and UMB groups showed greater reduction of microorganisms compared to the control group, for Gram negative (*E. coli*) and positive (*S. aureus*) bacteria in suspension condition. However, the urea group (UMB) did not perform better than the MB group, and urea was not, therefore, in this in vitro experiment, a determining factor in bacterial reduction, with microorganisms in suspension and not in biofilm.

MB has been widely used in aPDT. However, the mechanisms of action (Type I or Type II) are defined by their aggregation state. In this sense, the identification of aggregation, mechanisms of action and effectiveness against microorganisms, as well as the establishment of means and formulations that can favor the most effective mechanisms, is essential to improve the effectiveness of aPDT [7, 12, 13].

Despite the different available and effective treatments with antibiotics, alternative treatments are increasingly being sought, and their prescription is controlled by health regulatory agencies around the world [14]. Due to the indiscriminate use of antibiotic therapy, leading to microbial resistance, being the cause of morbidity and mortality due to the so-called "super-resistant" bacteria found mainly in the hospital environment, the systemic side effects of the drugs, the cost to the health system, initial action therapy, drug interaction in patients who use medications for underlying diseases is encouraged and the target of study of alternative therapies [5, 6, 11, 13, 14].

In alternative or adjuvant antimicrobial therapies, efficacy, speed, cost, low toxicity and safety in clinical application are sought. Within this context, aPDT comes across as a therapeutic resource in different clinical conditions in the health area [1, 6, 14].

Sodium dodecyl sulfate was the only one that improved the effectiveness of AM on aPDT in a culture of *C. Albicans* in which several vehicles were tested, in biofilm, including 1 mol/L urea [11]. This study shows that the possible breakdown of MB by urea, in this case, was not so efficient, in line with our work. But MB with urea had a shorter exposure time to totally eliminate *C. albicans* compared to MB without urea in another experiment [6]. The use of MB in gel form was tested to improve the generation of reactive oxygen species, comparing carbopol gel (CBP) and hydroxyethylcellulose (HEC) gel with 10% urea addition, 10% ethanol or water. The best results were obtained in CBP with 10% ethanol and 10% HEC in water [15]. In contrast, our study showed that urea did not obtain much lower results when added to MB.

MB was evaluated in deionized water and 0.9% saline solution for bacterial reduction in E. coli, with the best bacterial reduction being the MB associated with deionized water [16] in agreement with our experiment that used the mixture of MB with deionized water in the MB group. What should be considered is that the application of aPDT in vivo, as it contains secretions and several other substances that are not found in in vitro situations, may cause the urea disaggregating power to promote greater antimicrobial action, in addition to providing a decrease in the exposure time. As it is an in vitro study, in the

present study urea, despite exerting PF disaggregation, did not result in a better bacterial decrease than the group that used only MB.

5 Conclusions

The present study showed that aPDT with MB or MB with urea promoted superior microbial reduction compared to the control group, for Gram negative *(E. coli*) and Gram positive (*S. aureus)* bacteria in suspension. The microbial reduction of aPDT in the group with urea (UMB) was not higher than in the group without urea (MB). The disaggregation factor that urea has in phenothiazine PS, in this experiment, was not decisive to reduce the amount of microorganisms in suspension.

Conflict of Interest. The authors declare that there are no conflicts of interest in carrying out this study.

Statement of Animal Rights. The study was carried out after submission and approval by the Ethics Committee for the Use of Animals from Brazil University, protocol approval number IC18-19/016), following the precepts of ethics and animal welfare recommended by CONCEA.

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