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Antimicrobial effects of photodynamic therapy on *Staphylococcus aureus* biofilm grown on a specific acrylic resin surface for ocular prostheses

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ABSTRACT

Background: Photodynamic therapy (PDT) is a treatment for the specific control of oral biofilms. However, its effects on maxillofacial prostheses have been barely explored. In this study, we evaluated the antimicrobial effect of PDT using methylene blue (MB) and laser against a *Staphylococcus aureus* biofilm developed on the surface of scleral acrylic resin.

Methods: Sixty-six specimens of acrylic resin designed for ocular prostheses were fabricated in a disk-shaped format (3 × 10 mm). *S. aureus* biofilm was grown on the surface of the specimens for 24 h and the disks were then treated with MB at different concentrations (25, 50, 75 or 100 μg/mL), with or without PDT (GaAlAs diode laser; 660 nm; 100 mW; 9 J; 321.4 J.cm^{-2}; 3.5 W.cm^{-2} and 90 s). Control groups were treated with 2% chlorhexidine gluconate (CHX) or phosphate buffered saline. After the treatments, colony forming units (CFU) were counted and the samples were qualitatively evaluated by scanning electron microscopy (SEM). Data were analyzed descriptively and by nested ANOVA and the Tukey test (α = .05).

Results: PDT groups with MB concentrations at 75 and 100 μg/mL formed fewer CFU compared to the other groups ($P < 0.001$) and the 2% CHX group did not form any CFU. SEM images revealed that the surface of the polymers in these groups did not show bacterial colonies.

Conclusions: PDT significantly reduced *S. aureus* biofilm in the scleral acrylic resin when associated with an MB dilution of 75 μg/mL or higher. Thus, PDT can be a promising candidate for disinfecting ocular prostheses.

1. Introduction

Ocular prostheses provide important rehabilitation for individuals who have suffered total or partial loss of an orbit due to traumas, neoplasms or congenital diseases [1]. The treatment allows the improvement of the quality of life of these individuals since the eyes, in addition to permitting vision, also play an important role in communication and in the human relationships [2]. Currently, the material chosen for the fabrication of ocular prostheses is acrylic resin due to its advantages, i.e., availability on the market, low cost, easy handling, biocompatibility, and resistance to material degradation [3–5].

Biomaterials for ocular prostheses are susceptible to bacterial adhesion and colonization by potentially pathogenic microorganisms [6]. *Staphylococcus aureus* is considered to be the most important pathogen for prosthetic infections [6,7]. Lacrimal secretion and mucous and stagnant residues on the surface of the ocular prostheses may create an ideal culture environment for the growth of microorganisms [8]. In this respect, the control of biofilm formation in ocular prostheses is particularly important in order to maintain the health of the anophthalmic cavity and to prevent infections such as endophthalmitis [3,9]. Since

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acrylic resin is a thermosensitive material and should not be subjected to any disinfection procedure involving high temperatures, methods for immersion in chemical disinfectants are currently considered to be more suitable for ocular prostheses [10].

Among the disinfectant agents used in ocular prostheses, the chlorhexidine gluconate (CHX) solution has been shown to be effective against fungal and bacterial patogens [10]. Nevertheless, the continued use of CHX may alter the physical and mechanical properties of the acrylic resin, mainly roughness and microhardness [11,12]. Hence, acrylic resin may acquire an irregular surface, with minor imperfections such as marks and scratches that are uncomfortable for the users [13]. Moreover, CHX residues impregnating the acrylic bases of dentures have important genotoxic and cytotoxic effects directly on human oral cells such as gingival fibroblasts, endothelial cells and alveolar osteoblasts [14,15].

Antimicrobial photodynamic therapy (PDT) has been used as a current disinfection protocol in dentistry and is considered effective in reducing bacteria and/or yeasts in single or multispecies biofilms [15–17]. PDT is a procedure consisting of three components: a light source (at a wavelength corresponding to the dye absorption spectrum), a photosensitizer such as methylene blue (MB), and molecular oxygen [17,18]. The energy transfer from the activated photosensitizer to the available oxygen results in the formation of high concentrations of reactive oxygen species (ROS) such as singlet oxygen and free radicals. The resulting ROS are thus responsible for the bactericidal effect of PDT [15,18]. The use of PDT as an emerging strategy for the inactivation of important pathogenic bacteria and yeast such as *Candida albicans* on acrylic denture bases and alongside palatal soft tissues has been described in the current literature [19,20]. However, studies about the effects of PDT in reducing the microorganism content of ocular prostheses, as well as alternative protocols for periodic disinfection, are still lacking.

Therefore, the purpose of this *in vitro* study was to evaluate the effect of PDT on the *S. aureus* biofilm developed on the surface of a specific scleral acrylic resin used for the manufacture of ocular prosthesis. The null hypothesis was that the proposed PDT with different concentrations of an MB photosensitizer would not affect *S. aureus* viability when grown on acrylic resin specimens.

2. Materials and methods

2.1. Specimen preparation

Sixty-six disk-shaped specimens were fabricated from microwave-cured scleral acrylic resin (Onda-Cryl; Clássico Artigos Odontológicos Ltd., São Paulo, SP, Brazil) in a metal matrix (3 × 10 mm) according to previously published method [21]. In a dental flask (VIPI STG; VIPI Indústria, Comércio, Exportação e Importação de Produtos Odontológicos Ltd., São Paulo, SP, Brazil), this metal matrix was placed between two glass plates (80 × 35 × 3 mm) with a smooth surface (to mimic the smooth and polished surface of the ocular prostheses), both insulated with an alginate-based product (Cel-Lac, SSWhite Duflex, Rio de Janeiro, RJ, Brazil) in order to avoid acrylic resin adherence to the glass plates after polymerization. The proportions of the powder and polymer of acrylic resin were previously measured in specific containers, poured and mixed with a stainless-steel spatula (no. 36; SSWhite Duflex) according to the manufacturer's instructions. When the mixture reached the filamentous phase, it was inserted into the metal matrix, then the muffle was closed and pressed under a pressure of 40 lbf. The dental flask was closed with the specimens confined inside and was left to stand for 30 min for subsequent polymerization of the acrylic resin in a microwave oven for 3 × 10 min according to manufacturer's instructions. Specimens were subjected to ultrasound cleaning (1440DA, Odontobrás Ltd., São Paulo, SP, Brazil) and packed in envelopes for sterilization with ethylene oxide in order to eliminate possible remaining microorganisms.

2.2. Bacterial strain and growth condition

S. aureus reference strain (ATCC 6538 slime-positive) was used in this study [22]. The strain was kept as frozen stock with 10 % glycerol at −80 °C until use. A total of 100 μL of stock culture was mixed with brain heart infusion broth (BHI) (Difco, Sparks, MD, USA) supplemented with 1% glucose and incubated overnight at 37 °C in a biological oxygen demand (BOD) incubator.

Initially, growth curves were constructed with the purpose of identifying the number of hours necessary to achieve the greatest bacterial multiplication phase (log phase), which was determined by the optic density values. Starter cultures, 30 mL in BHI were then grown at 37 °C for 12 h (overnight). After this period, *S. aureus* cultures were adjusted halfway through the logarithmic phase, being diluted 10×, 100×, or 100× in BHI broth with 2× concentration and optical density was measured with a spectrophotometer in order to set the concentration of 0.01 at OD600. The amount of inoculated culture was calculated in order to obtain approximately 1×10^8 CFU/mL.

2.3. Biofilm growth on the disc surface

Specimens (n = 6 per group) were transferred individually to a 24-well microplate containing 800 μL of bacterial inoculum, corresponding to 10^8 CFU/mL of *S. aureus* in BHI supplemented with 1% glucose. Under these conditions, 800 μL is the minimum volume needed to completely cover the specimens, showing viable biofilm adhesion for the tests. The plates containing the specimens with the inoculum were incubated for 24 h at 37 °C and then transferred to a new 24-well culture plate containing 800 μL PBS and washed once. The solution was then aspirated and the specimens were submitted to the disinfection treatment for each experimental group.

2.4. Preparation of the MB solutions and the PDT protocol

MB (CI 52015, Vetec Química Fina Ltd., Rio de Janeiro, RJ, Brazil) was used as the photosensitizing agent. A stock solution was prepared at 500 μg/mL (in PBS) and diluted in PBS to obtain final concentrations of 25, 50, 75 and 100 μg/mL.

The PDT protocol was established as follows: each MB solution was applied to the specimens and, after 5 min (pre-irradiation time), MB was removed from all wells. The biofilm was then washed with PBS and subjected to irradiation using a continuos wave diode laser device (GaAlAs, Therapy EC, DMC Equipamentos Ltd., São Carlos, SP, Brazil), with the following parameters: 660 nm, 100 mW, 9 J, 321.4 J.cm^{-2}, 3.5 W.cm^{-2}, and 90 s, in punctual and contact mode (Fig. 1) [23]. The device power was checked with a power meter (LaserCheck; Coherent Inc., Palo Alto, CA, USA) before each experiment.

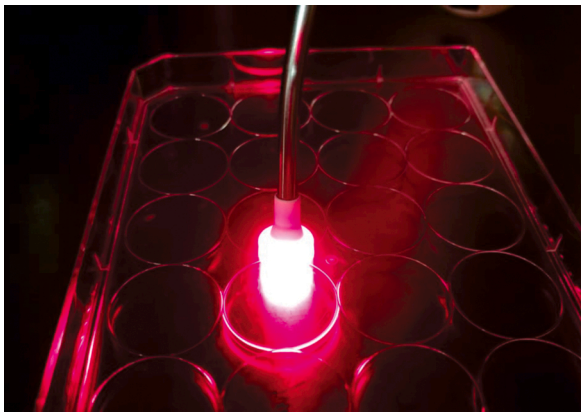

Fig. 1. Irradiation of specimens containing the biofilm using a continuous wave diode laser device.

2.5. Experimental groups

The specimens were randomly allocated to the following treatments:

(I) PBS as negative control (specimens were immersed for 10 min);
(II) 2% CHX solution (Maquira, Maringá, PR, Brazil) as positive control (specimens were immersed for 10 min);
(III) Diode laser (solely);
(IV) MB solutions at concentrations of 25 (MB25), 50 (MB50), 75 (MB75) or 100 (MB100) μg/mL (solely);
(V) PDT using MB solutions at concentrations of 25 (PTD25), 50 (PDT50), 75 (PDT75), and 100 (PDT100) μg/mL in association with a diode laser.

2.6. Biofilm CFU count

The solutions were aspirated and 800 μL of PBS was added to the wells to wash the specimens containing the biofilm and subsequently remove the weakly adhered cells. PBS solutions from the microplates were aspirated to remove excess products and 100 μL of PBS containing 0.005 % Tween 80 solution was added to the plate. The biofilm formed on the side of the specimens facing upwards was scraped with a sterile tip. The suspensions containing the biofilm were homogenized, 10 μL of each sample were serially diluted in 90 μL of PBS solution and the serial dilutions were seeded by the microdrop technique. One Petri dish was used for each treatment group and divided into five sections identified by the dilution performed, i.e., 10^{-2} to 10^{-6}. For each dilution section, three drops of 10 μL from the diluted suspensions were applied. All Petri dishes were incubated for 24 h at 37 °C in a BOD incubator. The number of CFU/mL was determined as the mean CFU count of the three drops for each dilution and multiplied by a correction factor and the inverse of the dilution.

2.7. Scanning electron microscopy (SEM) assessment

The surface of one specimen of material containing the biofilm and treated with the disinfectants proposed for the study was randomly selected, coated with a gold-palladium alloy immediately after surface treatment and placed on a carbon tape for imaging. Images were recorded at 1000× and 6000× magnification. The SEM unit (FEI Quanta 200 FEG) operated at 15 kV accelerating voltage and 10 mm working distance (WD).

2.8. Data analysis

Minitab software (version 17) was used for statistical analysis. The Box-Cox transformation was employed to obtain data according to the normal distribution for the Kolmogorov-Smirnov test. The transformation indicated that the natural logarithmic function (ln) applied to the data met the assumptions of ANOVA. Light dose and MB concentration are variables that reflect different aspects of the experiment. They were considered as variables in order to identify the individual effects of MB solutions, PDT protocol, and MB concentrations besides the effect of interactions among them. Three-way nested ANOVA was carried out to determine significant differences among groups of three variables (MB, PDT and concentration) and the significantly different values were compared by the Tukey-Kramer HSD test at the 5% level of significance.

3. Results

Specimens treated with CHX did not show *S. aureus* growth, showing complete inhibition of this bacterium. As the value was zero for the treatment with CHX, this result was not considered in the nested ANOVA.

There was a significant difference for all treatments (diode laser therapy, MB solutions and PDT) and the interaction among these factors ($P < 0.001$) (Table 1). The mean and standard deviation CFU values for each disinfection treatment are shown in Fig. 2. Lower CFU numbers were observed for specimens from the MB100 group (CFU = 1.13E+05), PDT75 group (CFU = 3.23E+04) and PDT100 group (CFU = 2.71E+04), with a statistically significant difference ($P < 0.001$) in relation to the PBS group (CFU = 2.57E+08). In addition, specimens treated with a diode laser and MB at the concentrations of 25 μg/mL and 50 μg/mL with or without laser, and at 75 μg/mL without laser showed lower CFU numbers compared to the PBS group ($P < 0.001$).

SEM images (Fig. 3) revealed that, under the current protocol, the PDT75 and PDT100 groups were efficient in eliminating the biofilm structure from the resin specimens. The biofilms exposed to the diode laser were less structured regarding the extracellular matrix. The combination of diode laser and the highest MB concentration tended to form isolated and more distant colonies. In the 2% CHX group, the images did not show structured biofilm formation.

4. Discussion

Attention has been closely paid to the CHX solution used as a periodic disinfectant in maxillofacial prostheses, since modifications in the structure of these materials have been reported [11,21,24]. Based on this background, the present study used an *in vitro* model to investigate whether PDT using MB as the photosensitizer acts as an antibacterial adjuvant against *S. aureus* on the surface of a scleral acrylic resin widely used in ocular prostheses. The null hypothesis that MB associated with laser would not affect the *S. aureus* biofilms grown on the scleral acrylic resin was rejected. Our main finding was that PDT significantly reduced the biofilm of *S. aureus* in the scleral acrylic resin when MB was used at a dilution of 75 μg/mL or higher. These findings were confirmed by SEM images which revealed the elimination of the biofilm structure from resin specimens.

The choice of a photosensitizer is an important factor for the successful application of PDT, and different dyes have been used to treat oral and maxillofacial conditions, each with its specific excitation wavelength [15,18]. MB is commonly used at different concentrations in clinical practice since it has shown beneficial properties for antimicrobial PDT such as low molecular weight (flowability), $^1O_2$ generation (high ROS), hydrophilicity, presence of a cationic form at physiological pH, low-cost effectiveness, and strong light absorption at 660 nm. Accordingly, MB presents excellent penetration of the cell membrane due to the capacity of its benzene ring to concentrate in the mitochondria, lysosomes and double-stranded DNA [17,18,25,26].

The results of the present study are promising, mainly because they open a new range of possibilities regarding alternative disinfecting procedures for ocular prostheses, since we observed a significant increase in antimicrobial activity compared to control. Since the authors

Table 1
Results of three-way analysis of variance (nested ANOVA) for log of the colony forming units per millimeter.

Factors	*df*	Sum of squares	Mean square	F	*P* value
MB	1	97.50	97.499	68.94	<0.001 *
PDT	1	109.80	109.877	77.69	<0.001 *
MB × PDT	1	24.99	24.994	17.67	<0.001 *
Concentration (PDT with different MB concentrations)	6	251.12	41.854	29.59	<0.001 *
Error	50	70.72	1.414	–	–
Total	59	531.82	–	–	–

MB, methylene blue; PDT, photodynamic therapy.
* $P < 0.05$ denotes a statistically significant difference.

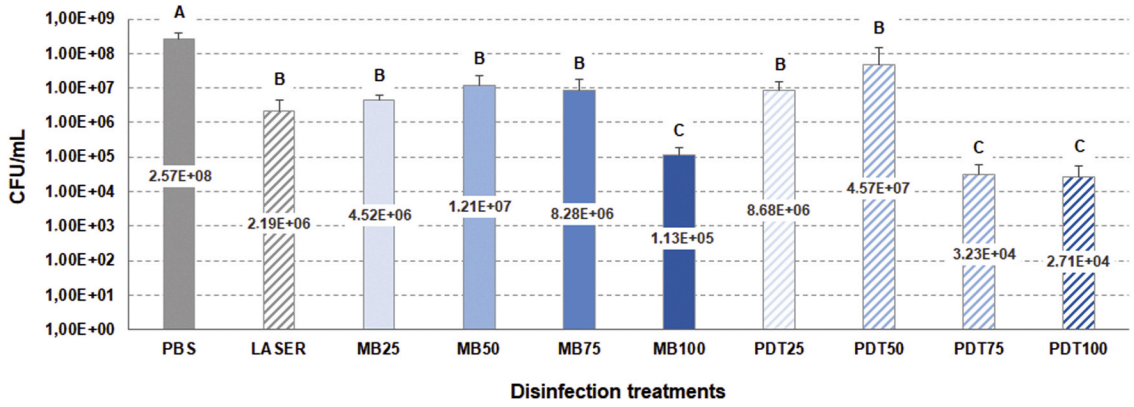

Fig. 2. Mean and standard deviation of the number of *Staphylococcus aureus* bacteria per milliliter adhered to the biofilm for each disinfection treatment. Different capital letters indicate a statistically significant difference among treatments ($P < 0.05$; nested ANOVA, Tukey test).

are unaware of previous studies that explored the effects of MB on scleral acrylic resin after PDT, it is important to discuss the studies that evaluated other similar biomaterials used in prosthodontics. Regarding the disinfection of denture surfaces, a recent systematic review considered PDT to be an auxiliary tool, mainly due to the reduction of microorganisms in acrylic resin samples [19]. However, the authors stated that there is some discrepancy in the definition of control groups between experimental studies [19]. It is known that the characteristics of surfaces can affect the adhesion and integrity of the biofilm and modify the effectiveness of the antimicrobial PDT [27,28]. For instance, in a study that evaluated the killing efficacy of an antimicrobial PDT protocol using 25 μg/mL purpurin as a photosensitizer against *Streptococcus mutans* biofilms grown on glass, denture acrylic and titanium, the authors demonstrated that biofilms adhered and cultured on titanium were the most difficult to disinfect, while acrylic dentures were the least difficult to disinfect [28]. In this regard, the variety observed in the survival of biofilm grown on different surfaces, whether in dental materials or in maxillofacial prostheses, may point out differences in the current composition of the biofilm matrix, which may be due to the properties of the surfaces on which they were developed [28,29].

We have followed a PDT protocol similar to that described by Guglielmi et al. [23], who reported the potential clinical use of PDT for the treatment of dental caries. In the current study, the photosensitizer was kept in contact with the samples for five minutes (pre-irradiation time). In fact, this period is important to allow drug uptake by the biological target. However, a major concern about the use of MB in scleral acrylic resin would be the residual blue pigmentation left by the photosensitizer. Certainly, further studies using MB as a photosensitizer should investigate whether there are changes in the optical parameters that could compromise the esthetic properties of these resinous materials.

Furthermore, it is noteworthy that the residual MB and the PDT procedure itself may have variable effects on the viability of eukaryotic cells. A previous study has demonstrated that 10 μmol.L^{-1} (approximately 0.31985 μg/mL) MB activated with 36 J.cm^{-2} light energy did not produce significant cytotoxicity to mouse fibroblasts [30]. In addition, Kashef et al. [31] and Darabpour et al. [32] documented that MB-mediated antibacterial PDT (163.8 J.cm^{-2} and 22.93 J.cm^{-2}, respectively) at distinct concentrations (i.e., at 2.5, 12.5 and 25 μg/mL) did not enhance cytotoxicity in human fibroblasts compared to the untreated group. In contrast, a combination of 1.0 mg.L^{-1} (or 1 μg/mL) MB associated with 7.5 J.cm^{-2} of LED significantly reduced cell viability, while MB and LED alone were harmless to fibroblasts. Nonetheless, MB-mediated antimicrobial PDT (7.5 J.cm-^{2} LED at 630 nm) induced cytotoxicity in mouse fibroblasts, with consequent activation of the Bcl-2 apoptosis signaling pathways [33]. In the present study, we did not evaluate the cytotoxicity of the PDT protocol on epithelial or mesenchymal cells. However, considering that the proposed PDT protocol for the disinfection of ocular prostheses would be used outside the anophtalmic cavity, a potential hazard to the host cells is unlikely.

In carious lesions, due to their more complex biofilm (multispecies), PDT may be an alternative approach to local microorganism reduction, using 0.01 % MB dye (10,000 μg/mL) and the same dosimetry as used in the present study [23]. On the other hand, *S. aureus* is an important bacterium considering its pathogenic potential, and may be resistant to elimination [34]. Considering that the protocol by Guglielmi and colleagues [23] was effective in complex biofilms of deep carious lesions, the idea was that it would be more effective against this isolated microbe. Herein, there was a decrease in CFU values when using MB at a dilution of 75 μg/mL or higher.

There are heterogeneities in the parameters that allow defining a protocol for antimicrobial PDT and photosensitizers in the disinfection of biomaterials [15,19]. Recently, an *in vitro* study evaluated PDT using a diode laser with a wavelength of 665 nm and MB as the photosensitizer to eradicate Gram-positive and Gram-negative bacteria that cause periprosthetic joint infection [35]. The authors demonstrated that Staphylococci were eradicated at the lowest concentration of 0.1 mM MB (around 31.9 μg/mL). It was also observed that when the highest laser doses were used, i.e., energy densities greater than 35 J.cm^{-2} and irradiances greater than 35 mW.cm^{-2}, *Staphylococcus epidermidis* was eradicated [35]. In the present study, we employed a diode laser with a wavelength of 660 nm, irradiance of 3.5 W.cm^{-2}, and energy density of 321.4 J.cm^{-2}. We also found optimal concentrations of 75 μg/mL and 100 μg/mL MB, since few numbers of CFU were observed. On this basis, these findings agree with previous studies that reported morphological and pattern features of *S. aureus* biofilms formed on the surfaces of different materials and treated with PDT [36,37].

S. aureus was chosen for this study since it has been extensively used in models for bacterial adhesion due to its great importance in biofilm formation. Although the skin is normally colonized by *S. aureus*, the infection is not exogenous in most cases. In addition, *S. aureus* is a common pathogen found in prosthetic infections [6,36,37]. Moreover, the ocular microbial biofilm of individuals with endophtalmitis consists of different microbial species such as *S. epidermidis*, *Staphylococcus warneri*, *Enterococcus faecalis*, *Enterobacter asburiae*, *Stenotrophomonas maltophilia*, *C. albicans*, and others [6,9]. The use of PDT on single- or multi-species biofilm formation can be an alternative therapy for reducing or eliminating undesirable pathogens that, at least transiently, reside on the materials used in maxillofacial prostheses.

Based on the results of this *in vitro* study, PDT demonstrated efficacy in the disinfection of *S. aureus* biofilms in scleral acrylic resin. Among the groups studied, 2% CHX showed total removal of *S. aureus* biofilm from the resin specimens. However, there was an important decrease in CFU values with all alternative treatments proposed compared to

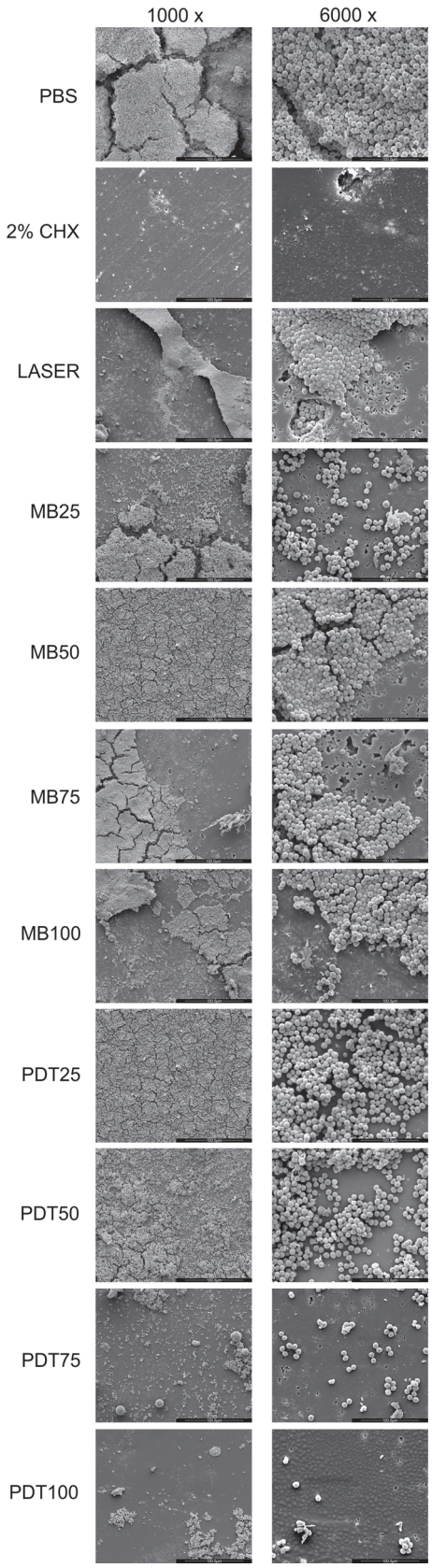

Fig. 3. Scanning electron microscopy images at 1000× (scale bar =50 μm), 6000× (scale bar =10 μm) of specimens of acrylic resin specific for ocular prostheses colonized by *Staphylococcus aureus* biofilm, immersed in different disinfectant treatments. H_2O was used as the negative control. WD = 10 mm; 15 kV.

control, particularly when higher MB concentrations were used. Thus, the use of MB at a dilution equal to or greater than 75 μg/mL, if associated with light, can be a promising auxiliary tool for the disinfection of ocular prostheses.

Declaration of Competing Interest

The authors report no declarations of interest.

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