

## Photodynamic therapy of oral *Candida* infection in a mouse model



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### ABSTRACT

Species of the fungal genus *Candida*, can cause oral candidiasis especially in immunosuppressed patients. Many studies have investigated the use of photodynamic therapy (PDT) to kill fungi *in vitro*, but this approach has seldom been reported in animal models of infection. This study investigated the effects of PDT on *Candida albicans* as biofilms grown *in vitro* and also in an immunosuppressed mouse model of oral candidiasis infection. We used a luciferase-expressing strain that allowed non-invasive monitoring of the infection by bioluminescence imaging. The phenothiazinium salts, methylene blue (MB) and new methylene blue (NMB) were used as photosensitizers (PS), combined or not with potassium iodide (KI), and red laser (660 nm) at four different light doses (10 J, 20 J, 40 J and 60 J). The best *in vitro* log reduction of CFU/ml on biofilm grown cells was: MB plus KI with 40 J (2.31 log;  $p < 0.001$ ); and NMB without KI with 60 J (1.77 log;  $p < 0.001$ ). These conditions were chosen for treating the *in vivo* model of oral *Candida* infection. After 5 days of treatment the disease was practically eradicated, especially using MB plus KI with 40 J. This study suggests that KI can potentiate PDT of fungal infection using MB (but not NMB) and could be a promising new approach for the treatment of oral candidiasis.

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### 1. Introduction

A leading cause of opportunistic infections are fungal species belonging to the *Candida* genus, with *Candida albicans* ranking as the most prevalent causative agent of candidiasis and candidemia around the world [1–6]. This trend has been observed over the past decade and is still the case, even in developed countries such as the United States, Denmark, Norway, and Finland [3]. *C. albicans* is a human commensal organism, and can colonize the skin, mucosal surfaces, gastrointestinal tract and the female genitourinary tract [7]. One important factor that contributes to the pathogenesis of candidiasis is biofilm formation, as *C. albicans* has the ability to form biofilms on both inert and biological surfaces [8]. These biofilms are typically surrounded by an extracellular polymeric substance [9] which effectively shelters the fungal cells against adverse environmental conditions, including host defense mechanisms and the action of antifungal drugs [10]. Research and development of new antifungal agents is complicated by the paucity of selective microbial targets, since fungi are also eukaryotic cells [11]. In addition, drug resistance of *C. albicans* against antifungals such as azoles and echinocandins represents an increasing problem [12].

Therefore, novel antifungal and antibiofilm drugs against these unmanageable infections are urgently needed [10,11]. Photodynamic therapy (PDT) has emerged as a promising modality due to its effectiveness against a broad range of species of microorganisms regardless of drug resistance [13]. In this approach, a photosensitizing agent or dye, which is activated by a light source at a specific wavelength in the presence of oxygen, resulting in the production of reactive oxygen species (ROS) and free radicals, is administered into the infected area. These photogenerated ROS disrupt the *Candida* cytoplasmic membrane and cause an increase in cellular permeability and subsequent damage to intracellular targets. It has been suggested that this oxidative stress might compromise virulence factors of the microorganism, such as the capacity to adhere to host epithelial cells, production of proteinases, reduction of biofilms and formation of germ tubes [14–20].

Mice have been used as models to study oral *C. albicans* infections and offer some advantages over a rat model in that mice do not harbor *Candida* spp. in their buccal cavity and therefore do not elicit an adaptive immune response against this yeast [21,22]. Optimally, the detection of light from small animals containing bioluminescent cells can be achieved using a charge-coupled device (CCD) based imaging system [23] allowing real-time non-invasive monitoring of the infection.

We have found no studies in the literature that monitored oral *C. albicans* infections in mice by bioluminescence imaging after use of photodynamic therapy, which could be a therapeutic option for the treatment of oral candidiasis. PDT could be a sole treatment option, or could be used as a coadjuvant to antifungal chemotherapy, improving

Abbreviations: PDT, photodynamic therapy; MB, methylene blue; NMB, new methylene blue; PS, photosensitizers; KI, potassium iodide.

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the treatment of oral infections by increasing therapeutic efficacy and by reducing costs.

For these reasons the objective of the present study was to monitor oral candidiasis produced by a luciferase-expressing *C. albicans* strain (CEC 749) in immunosuppressed mice using bioluminescence imaging before and after PDT using four different doses of red light (10 J, 20 J, 40 J and 60 J) with methylene blue or new methylene blue combined or not with potassium iodide to possibly potentiate the PDT effect. We have recently reported that addition of KI could potentiate antimicrobial PDT *in vitro* and *in vivo* mediated by MB [24], and also PDT mediated by cationic fullerenes in a mouse model of a wound abrasion infected by *Acinetobacter baumannii* [25].

## 2. Materials and Methods

### 2.1. Ethics Committee

All animal procedures in this study were approved by the Subcommittee on Research Animal Care (IACUC) of the Massachusetts General Hospital under the protocol (2015N000099) and were in accordance with the guidelines of the National Institutes of Health (NIH).

### 2.2. Cells and Culture Conditions

A luciferase-expressing *C. albicans* strain (CEC 749) was used in this study [26]. The luciferase reporter was constructed by fusing a synthetic, codon-optimized version of the *Gaussia princeps* luciferase gene (gLUC59) to *C. albicans* PGA59, which encodes a glycosylphosphatidylinositol linked cell wall protein. Luciferase expressed from this PGA59-gLUC fusion was localized at the *C. albicans* cell surface [26], where it can easily come into contact with its substrate coelenterazine (Gold Biotechnology, Inc., Charlestown, MA, USA) in intact cells. This system allows bioluminescence imaging of *C. albicans* infections after simple addition of coelenterazine solution to the surface of the infected tissue [26]. This approach also allows the detection of luciferase in both hyphae and yeast cells [26,27], given the central importance of the yeast-hyphal transition in *C. albicans* virulence [28].

*C. albicans* was routinely grown at 30 °C on yeast extract-peptone-dextrose (YPD) agar and subcultured in YPD medium. The inoculum was prepared by growing the *C. albicans* cells in 3 ml YPD broth overnight at 30 °C with shaking at 130 rpm in a C24 incubator/shaker (New Brunswick Scientific, Edison, NJ). The broths were then centrifuged at 12,000 rpm for 3 min (centrifuge 5417 C; Eppendorf, Hamburg, Germany) and resuspended in 0.5 ml of phosphate-buffered saline (PBS) or YPD. The concentrations were then adjusted to the appropriate values for the experiment by measuring the optical density at 570 nm (OD570) of the *C. albicans* suspensions, using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific). An OD570 of 0.65 correspond to a fungal concentration of 10<sup>7</sup> CFU/ml in PBS (*in vivo* test) or in YPD media (*in vitro* test).

### 2.3. Photosensitizers and Light Source

The phenothiazinium salts methylene blue (MB) (M9140-25G; Sigma), and new methylene blue (NMB) (556416-1G; Sigma) with or without potassium iodide (KI) were used as PSs in the study. The dye contents of MB and NMB were approximately 85% and 70%, respectively. These photosensitizers were selected by a preliminary study based on the criterion that the concentration produced an effective PDT effect with minimal dark toxicity [29]. Stock PS solutions (10 mM) were prepared by dissolution of the PS powders in sterilized water and were stored in the dark at 4 °C for no longer than 14 days.

The light source used in this study was emitted by a diode laser (Arroyo Instruments, LLC, San Luis Obispo, CA, USA) at a wavelength of 660 nm, at a power of 100 mW, delivering 10 J, or 20 J, or 40 J, or 60 J.

All parameters are given in Table 1. For *in vitro* experiments, the laser beam was expanded by a lens (model LM2-B) to form a spot diameter 4.7 cm delivered without contact with the plates (distance of 7 cm). For *in vivo* experiments, the same laser beam was delivered using an optic fiber without a lens, without contact with mouse tongue (distance of 5 cm), covering a spot area of 0.38 cm<sup>2</sup>.

Both the best sets of conditions from *in vitro* experiments using PDT with NMB (NMB 60 J) and MB (MB/KI 40 J) were chosen to be applied on the *in vivo* model of oral candidiasis. It is important to highlight that the light parameters were kept the same from the *in vitro* experiments (wavelength, total power and irradiation time). The optical power reaching the plates (*in vitro*) and the mouse tongue (*in vivo*) was measured using an optical energy meter PM100D Thorlabs® fitted with a sensor S310C (3.14 cm<sup>2</sup>).

### 2.4. In vitro Photodynamic Therapy

In order to grow the biofilms a 250 µl aliquot of the suspension (10<sup>7</sup> CFU/ml in YPD) was pipetted into each well of a 96-well flat-bottom microtiter plate. The plate was incubated for 1.5 h at 30 °C in a shaker at 75 rpm for the initial adhesion phase. After this period, the wells were washed with 250 µl of PBS to remove loosely adhered cells. A 250 µl aliquot of YPD was then pipetted into each washed well, and the plate was incubated at 30 °C in a shaker at 75 rpm for 48 h. The broth was changed after 24 h. The plate with biofilms formed by *C. albicans* was then washed with 250 µl of PBS to remove loosely adherent cells.

The biofilm formed by each strain was immersed in 100 µl of a solution of each photosensitizer (PS) or PBS for 10 min (pre-irradiation time). Subsequently, the suspended plate was irradiated according to the groups:

- P – L –: received PBS in absence of light. (n = 6).
- P – L +: the effect of the light source only with different doses 10, 20, 40 and 60 J. (n = 6 for each dose).
- P + L –: the effect of the PS only [MB (100 µM) or NMB (100 µM) and these PSs combined with KI (100 mM)]. (n = 6 for each PS).
- P + L +: treatment with each PS [MB (100 µM) or NMB (100 µM) and these PSs combined with KI (100 mM)] and each dose of light (10, 20, 40 and 60 J). (n = 6 for each PS and dose of light).

After this, each well received 100 µl of PBS and the biofilm was disrupted by homogenizing for 1 min using an ultrasonic homogenizer. The suspensions in the wells were considered to be a dilution factor of 10<sup>-1</sup>. 5 serial dilutions were then made using each original 10<sup>-1</sup> dilution, and aliquots of 10 µl were seeded onto YPD agar plates [30] that were then incubated at 30 °C for 24 h. After the incubation period, the CFU/ml values of each plate were determined.

**Table 1**  
Parameters of light for photodynamic therapy (PDT).

<i>In vitro</i>	<i>In vivo</i>
Wavelength (nm): 660	Wavelength (nm): 660
Pulse frequency: continuous	Pulse frequency: continuous
Laser power (mW): 100	Laser power (mW): 100
Irradiation time (sec): 100/200/400/600	Irradiation time (sec): 400/600
Total energy (J): 10/20/40/60	Total energy (J): 40/60
Energy density (J/cm <sup>2</sup> ): 3.18/6.36/12.73/19.10	Energy density (J/cm <sup>2</sup> ): 105.26/157.89
Power density (mW/cm <sup>2</sup> ): 31.84	Power density (mW/cm <sup>2</sup> ): 263.15
Application mode: without contact	Application mode: without contact

## 2.5. Induction of Experimental Candidiasis

A total of 15 adult male BALB/c mice (Charles River Laboratories, Wilmington, MA), 7 to 8 weeks old, weighing 17 to 21 g and with no *Candida* in their buccal cavities, were used in all experiments. The animals were housed with access to food and water *ad libitum* and were maintained on a 12 h light/dark cycle under a room temperature of 21 °C.

The methodology described by Takakura et al. [31] was used to induce experimental candidiasis with some modifications. Briefly, the animals were pre-treated with two separate doses of methylprednisolone acetate (Depo-Medrol, McKesson Medical-Surgical, Inc., Northborough, Boston) in order to create a temporary state of immunosuppression. 150 mg kg<sup>-1</sup> of Depo-Medrol were injected intramuscular (i.m.) 1 day before infection with *Candida*, followed by a second dose of 150 mg kg<sup>-1</sup> injected i.m. 3 days after infection with *Candida*. Tetracycline chloride (Terramicina, Laboratórios Pfizer Ltda., Guarulhos, SP, Brazil) was administered in the drinking water at a concentration of 0.83 mg/ml beginning 1 day before infection and maintained throughout the experiment. Before *C. albicans* infection, the mice were anesthetized by intraperitoneal (i.p.) injection of a ketamine–xylazine cocktail.

A sterile swab soaked in the *C. albicans* suspension (luciferase-expressing *C. albicans* concentration of 10<sup>7</sup> CFU/ml in PBS) was used to inoculate the sedated mice by rubbing the swab for 1 min on the tongue dorsum.

## 2.6. In Vivo Photodynamic Therapy

Twenty-four hours after the second injection of Depo-Medrol, PDT was conducted on the animals under anesthesia produced by intraperitoneal injection of a mixture of ketamine/xylazine (100 mg/kg; 10:1 ratio). The mice were divided into three groups: a) MB/KI + 40 J+: sensitization with MB (1 mM) with KI (1 M) and 40 J of light irradiation (n = 5); b) NMB + 60 J+: sensitization with NMB (1 mM) without KI combined with 60 J of light irradiation (n = 5); and c) P–L–: control group not subjected to sensitization with PS or light irradiation (n = 5). The groups “a” and “b” were chosen according to the best results on the *in vitro* tests.

Before light irradiation, a 10 µl volume of respective PS (1 mM) solution were pipetted onto the tongue dorsum and PS was then incubated for 5 min in the dark. After this time, the group “a” received 5 µl of KI solution (1 M) onto the tongue. The solution of the PS was liquid and although a small amount of the PS had trickled down, the majority of the relatively small drop adhered to the mouse tongue that turned blue before the application of light (Fig. 1). The *in vivo* dark toxicity of the dye was quantified by bioluminescence imaging. To take these images, 10 µl of coelenterazine (500 µg/ml in 1:9 methanol-PBS; Gold Biotechnology, Inc., St. Louis, MO, USA) [26] were topically smeared onto the surface of each infected tongue. The 10% methanol present in the solvent was not toxic to the *Candida* cells. Mice received light exposure of 40 J or 60 J, according to the group, with bioluminescence imaging taking place after each exposure of light. The light was delivered at an irradiance of 263.15 mW/cm<sup>2</sup> (groups “a” and “b”). The control group (“c”) received only coelenterazine in order to carry out the bioluminescence imaging.

To record the time course of the extent of fungal infection, fungal bioluminescence images from the mouse tongue infection was measured daily after each PDT during the 5 days of daily treatment. Euthanasia was performed with an overdose of i.p. injection of a ketamine–xylazine cocktail. The tongues were longitudinally cut in half, and one-half of each organ was fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin, sectioned (5 to 7 µm thick), and stained with hematoxylin and eosin (HE) and Periodic Acid-Schiff (PAS) for histologic analysis.

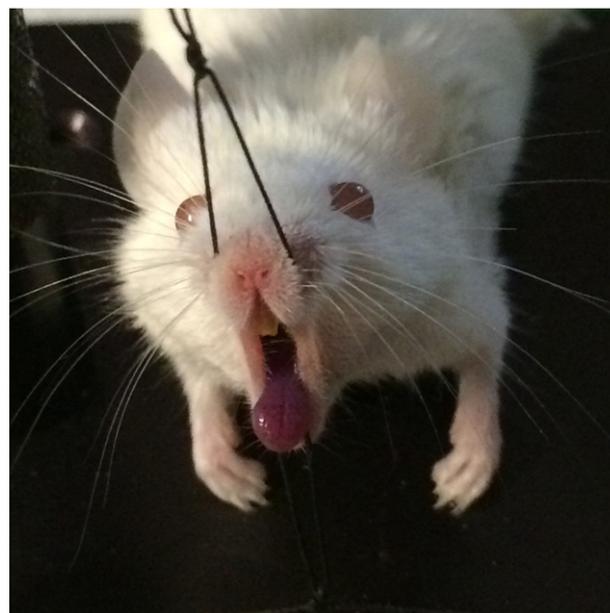


Fig. 1. Mouse tongue turned blue after the application of photosensitizer.

## 2.7. Bioluminescence Imaging

The IVIS® Lumina Series III (PerkinElmer, Inc., Waltham, MA, USA) was used for bioluminescence imaging. Using photon counting mode, an image can be obtained by detecting and integrating individual photons emitted by the fungal cells. Prior to PDT and imaging, mice were anesthetized by i.p. injections of ketamine–xylazine cocktail. Mice were then placed on an adjustable stage in the imaging chamber, and the tongue infected was positioned directly under the camera. A grayscale background image of each tongue was made, and this was followed by a bioluminescence image of the same region displayed in a false-color scale ranging from red (most intense) to blue (least intense) and superimposed on the grayscale image. The signal from the bioluminescence image was quantified as region of interest (ROI) with absolute calibrated data in: photons s<sup>-1</sup> cm<sup>-2</sup> sr<sup>-1</sup>, using the IVIS software.

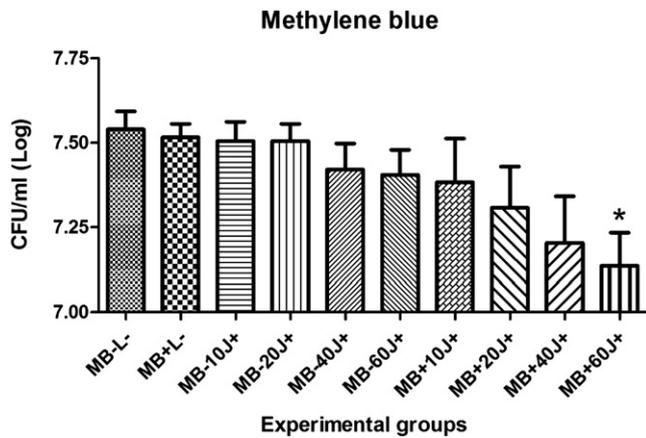
## 2.8. Statistical Analysis

The data for CFU/ml were converted to logarithmic form and analyzed by one-way analysis of variance (ANOVA) and Tukey HSD post hoc test. The CFU/ml reduction for *C. albicans* biofilms were calculated comparing each group (P + L–; P–L+; P + L+) and its subgroups, with the control group (P–L–).

In a two-dimensional coordinate system, the region of interest (ROI) data, which represent the time courses of fungal luminescence of the tongue infected were calculated using numerical integration. Differences in ROI among the control, NMB 60 J and MB/KI 40 J groups in the *in vivo* study were compared day by day using also ANOVA and Tukey HSD post hoc test. Significance was set at  $p < 0.05$ .

## 3. Results

Results for the PDT of *C. albicans* biofilms *in vitro* using MB or NMB as PSs, with or without addition of KI and the different light doses are shown in Figs. 2, 3, 4 and 5. Only four of twenty groups were not effective (MB + 10 J, MB + 20 J, MB + 40 J and NMB + 10 J). In all other groups we had a significant reduction. It should be noted that for planktonic cells an antimicrobial effect requires at least 99.9% of *Candida* killing. However for biofilms less than 3 logs of killing is needed to be

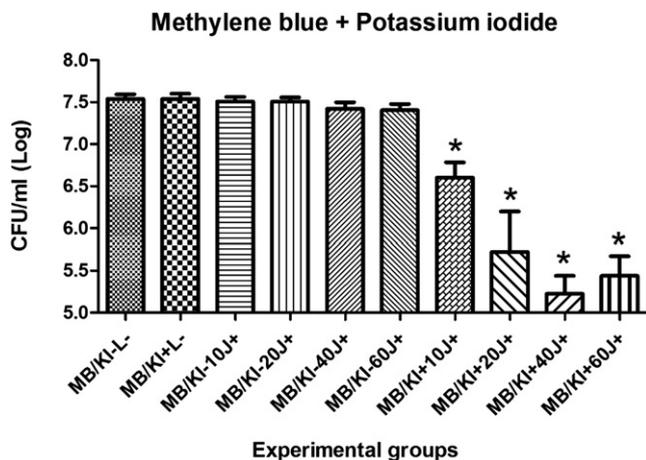


**Fig. 3.** Mean values and standard deviation of CFU/ml ( $\log_{10}$ ) of *C. albicans* biofilms exposed to the following treatments: MB (100  $\mu$ M) combined with KI (100 mM) and 10, 20, 40 or 60 J of laser doses in comparison with their respective controls, the effect of light alone, the effect of PS alone, and absolute control (no PS, no light). To analyze significance, one-way analysis of variance (ANOVA) and Tukey HSD post hoc test. The CFU/ml reduction for *C. albicans* biofilms were calculated comparing each group (P+L-; P-L+; P+L+) and its subgroups, with the control group (P-L-). Values followed by asterisk differed significantly among the experimental conditions ( $p < 0.05$ ).

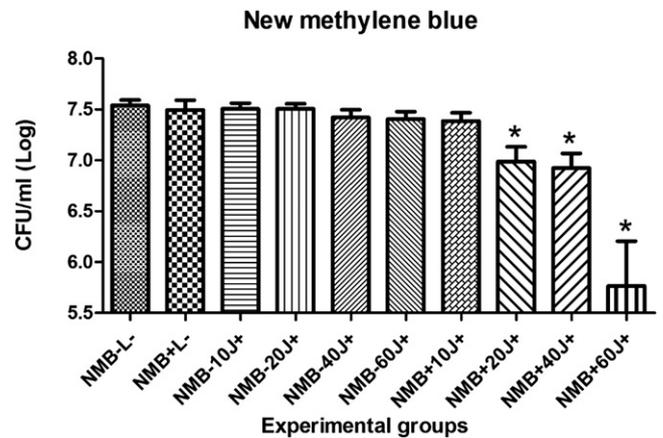
antimicrobial. The best log reductions of colony-forming units per milliliter (CFU/ml) were: MB (100  $\mu$ M) combined with KI (100 mM) and 40 J; and NMB (100  $\mu$ M) without KI with 60 J of irradiation, with log reductions ( $p < 0.001$ ) of 2.31 and 1.77, respectively. These two sets of conditions were used in the *in vivo* infection model.

Fig. 6 shows a comparison among the four treatments used in this study. The group MB plus KI showed significantly more fungal killing compared to MB alone at all doses of light utilized (10, 20, 40 and 60 J) and with NMB alone with 10, 20 and 40 J of light irradiation. MB plus KI also had a significant difference compared to NMB plus KI with 40 J, and MB alone had significantly more killing compared to NMB alone when these groups were irradiated with 60 J.

After 4 days of *Candida* inoculation (day 1 of treatment) a strong positive *Candida* bioluminescence signal was achieved with the peak occurring after 6 days for the control group (Fig. 7C). In the majority of



**Fig. 5.** Mean values and standard deviation of CFU/ml ( $\log_{10}$ ) of *C. albicans* biofilms exposed to the following treatments: NMB (100  $\mu$ M) combined with KI (100 mM) and 10, 20, 40 or 60 J of laser doses in comparison with their respective controls, the effect of light alone, the effect of PS alone, and absolute control (no PS, no light). To analyze significance, one-way analysis of variance (ANOVA) and Tukey HSD post hoc test. The CFU/ml reduction for *C. albicans* biofilms were calculated comparing each group (P+L-; P-L+; P+L+) and its subgroups, with the control group (P-L-). Values followed by asterisk differed significantly among the experimental conditions ( $p < 0.05$ ).

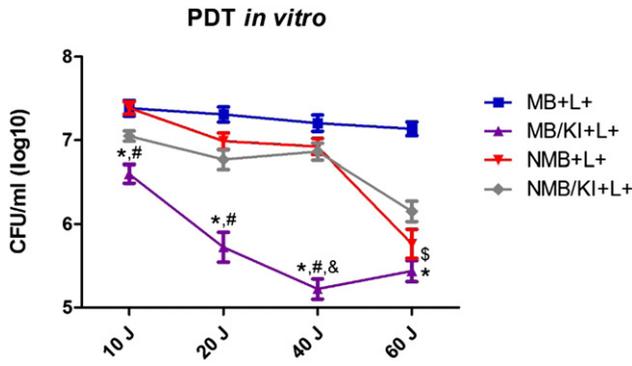


**Fig. 4.** Mean values and standard deviation of CFU/ml ( $\log_{10}$ ) of *C. albicans* biofilms exposed to the following treatments: NMB (100  $\mu$ M) with 10, 20, 40 or 60 J of laser doses in comparison with their respective controls, the effect of light alone, the effect of PS alone, and absolute control (no PS, no light). To analyze significance, one-way analysis of variance (ANOVA) and Tukey HSD post hoc test. The CFU/ml reduction for *C. albicans* biofilms were calculated comparing each group (P+L-; P-L+; P+L+) and its subgroups, with the control group (P-L-). Values followed by asterisk differed significantly among the experimental conditions ( $p < 0.05$ ).

animals subjected to immunosuppression and *Candida* inoculation, white lesions typical of pseudomembranous candidiasis were observed (Fig. 7A).

Total loss of bioluminescence signal was seen in two animals on the fifth day of treatment (Fig. 7B) with NMB (1 mM), as can be seen in the example in Fig. 7C. Fig. 7D shows that both PSs were effective on the treatment of oral candidiasis. No difference was found between the two PSs ( $p > 0.05$  for all days of treatment).

A representative set of periodic-acid-Schiff (PAS) and hematoxylin and eosin (HE) stained images from the infected tongue on day 5 post infection are shown in Fig. 8. These images demonstrated the presence of numerous *C. albicans* cells in the form of yeast and multicellular hyphal filaments in the control group. While all yeast cells were located on the surface of the keratinized epithelial layer of the tongue, the hyphal filaments branched out in every direction into the tissue. A host



**Fig. 6.** Mean values and standard deviation of CFU/ml (log<sub>10</sub>) of *C. albicans* biofilms exposed to the following treatments: MB (100 μM), MB (100 μM) combined with KI (100 mM), NMB (100 μM) and NMB (100 μM) combined with KI (100 mM); all treated with 10, 20, 40 or 60 J of laser light. To analyze significance, a ANOVA and Tukey HSD post hoc test were used. Values followed by asterisk had difference between MB and MB plus KI, values followed by hash had difference between NMB and MB plus KI, values followed by “&” had difference between MB plus KI and NMB plus KI and values followed by “\$” had difference between MB and NMB. (p < 0.05).

response to *C. albicans* infection, characterized by an inflammatory infiltrate, was also observed. Several lesions in the epithelial tissue were also observed, including epithelial hyperplasia, basal cell layer disorganization, exocytosis, loss of filiform papillae, hyperkeratosis and intraepithelial micro abscesses development. The figure also demonstrated that in the PDT treated groups the surfaces of the tongue appeared intact showing well-defined layers without visible yeasts or hyphal filaments, and without any lesions in the epithelial tissue.

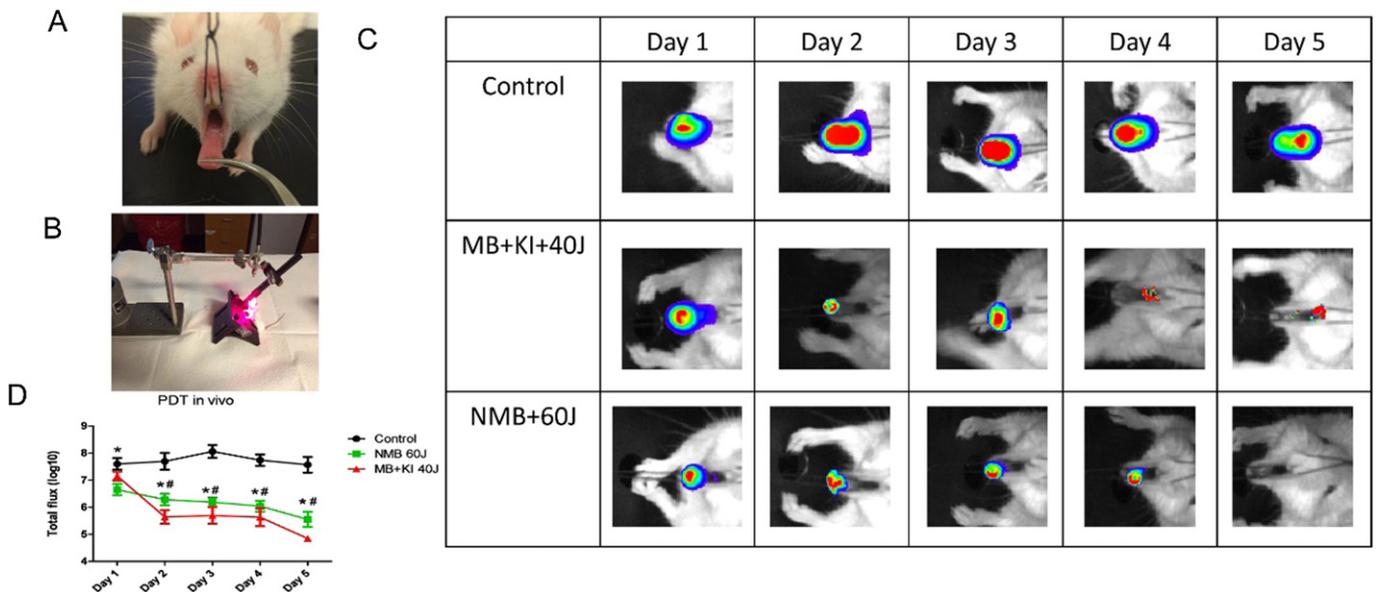
**4. Discussion**

*C. albicans* is the most frequent fungal species isolated from superficial and invasive fungal infections and systemic infections are associated

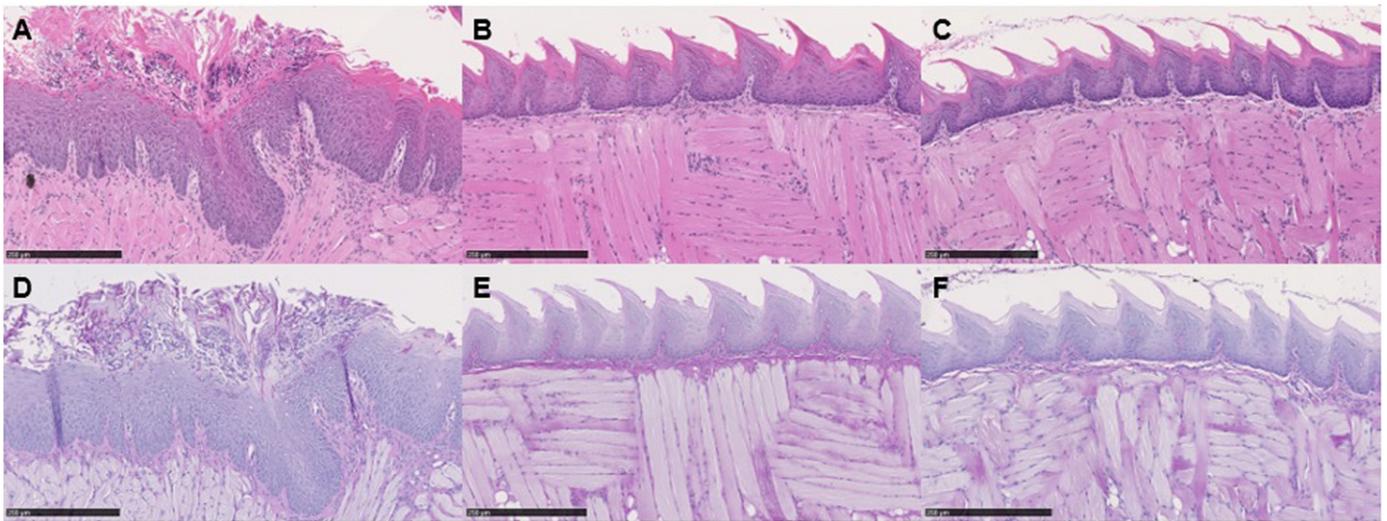
with high rates of mortality. Since there is an increasing number of strains of this microorganism that are resistant to antifungal agents, studies are needed to evaluate the effect of photodynamic therapy on oral candidiasis infections. In this study, we used a low light imaging charge-coupled device (CCD) camera to non-invasively monitor the results in real time. The treatment of candidiasis continues to be a challenge in dentistry. In this respect, photodynamic therapy has shown promising results as a coadjuvant treatment for this disease.

Schelenz et al. [32] isolated 269 strains of *Candida* spp. from the buccal cavity of patients with hematological malignancies or head and neck solid tumors. The authors evaluated the sensitivity profile of these strains to antifungal agents and found resistance to fluconazole (4.5%), itraconazole (11.7%), ketoconazole (11.3%), voriconazole (0.75%), and caspofungin (41.1%); however, all strains were susceptible to amphotericin B. Thus, amphotericin B has been used in treatment of *Candida* infections caused by fluconazole-resistant strains; however, amphotericin B can have substantial side effects, and a few cases of resistance have been reported [33]. For these reasons PDT is a new approach being developed for the treatment of candidiasis and the aim of this study was to study the effects of PDT both *in vitro* and *in vivo*.

Zhang et al. [25] synthesized C60-fullerene (LC16) bearing a decaquaternary chain and deca-tertiary-amino groups that facilitated electron-transfer reactions via the photoexcited fullerene. Addition of the harmless salt, potassium iodide (KI) (10 mM) potentiated the ultraviolet A (UVA) or white light-mediated killing of Gram-negative bacteria *A. baumannii*, Gram-positive methicillin-resistant *Staphylococcus aureus* and fungal yeast *C. albicans* by 1–2+ logs. A mouse model of a skin abrasion infected with bioluminescent *A. baumannii* gave increased loss of bioluminescence when iodide (10 mM) was combined with LC16 and UVA/white light. In a study from the same group, Vecchio et al. [24] added KI to MB and observed a consistent increase of red light-mediated bacterial killing of Gram-positive and Gram-negative species *in vitro* and *in vivo*. *In vivo*, they also observed less bacterial recurrence in wounds in the days post-treatment. They proposed that the mechanism of action was probably due to formation of reactive iodine species that



**Fig. 7.** (A) White patches observed on the tongue dorsum of a mouse without treatment. (B) Representative image during the applying of PDT. (C) Successive bioluminescence images of three representative mice tongues infected with 10<sup>7</sup> CFU/ml of *C. albicans* and treated with PDT during 5 days, starting on day 4 after fungal inoculation, in comparison with the control group that did not receive light and PS. PDT was carried out with a combination of MB (1 mM) together with KI (1 M) and 40 J of red laser; and using NMB (1 mM) with 60 J of light. (D) Mean values and standard deviation of total photon flux (log<sub>10</sub>) of bioluminescent *C. albicans* infection exposed to the following treatments daily during 5 days: MB (1 mM) combined with KI (1 M) and 40 J of red laser; and NMB (1 mM) with 60 J of light in comparison with the control group with absence of light and PS. To analyze significance, a ANOVA and Tukey HSD post hoc test were used comparing day by day of treatment. Values followed by hash had difference statistic between MB/KI + 40 J+ and the control group and values followed by asterisk had significant difference between NMB + 60 J+ and the control group (p < 0.05).



**Fig. 8.** Representative hematoxylin and eosin (8A, 8B and 8C) and periodic acid-Schiff (8D, 8E and 8F) tongue biopsy specimen taken from a mouse tongue infected with  $10^7$  CFU/ml of *C. albicans*, showing the presence of lesions in the epithelial tissue including epithelial hyperplasia, basal cell layer disorganization, exocytosis, loss of filiform papillae, and hyperkeratosis (8A) and the presence of yeasts and hyphal filaments (8D); in comparison with the groups that received treatment with NMB (100 mM) plus 60 J light dose (8B and 8E); and MB (100 mM) combined with KI (1 M) plus 40 J light dose (8C and 8F) that shows the tissues to be in intact condition. Biopsies were taken on day 5 post-infection. Bar, 250  $\mu$ m.

are produced quickly with a short lifetime. In our study MB (100  $\mu$ M) was combined with KI (100 mM) and four different doses of light (10, 20, 40 and 60 J) against *C. albicans* biofilms grown *in vitro*. When MB was used without KI the best log reduction was found with 60 J of light energy while the MB/KI solution achieved 2.31 of log reduction with 40 J of irradiation. This last result was the best one in all *in vitro* tests of our study and was chosen to apply to the oral *Candida* infection.

Freire et al. [34] compared Rose Bengal (RB) with Eosin Y (2,4,5,7 tetrabromofluorescein) assessing their potency for photoinactivation of *C. albicans* biofilms. After culturing these biofilms for 48 h, treatment was done with 200  $\mu$ M RB or Eosin Y for 5 min as a pre-irradiation period and subsequent illumination with a green LED (90 mW;  $532 \pm 10$  nm) for 180 s (light dose: 42.63 J/cm<sup>2</sup>; applied energy: 16.2 J), which resulted in inactivation rates of 0.22 and 0.45 log<sub>10</sub> for RB and Eosin Y, respectively. In our study MB had much better log reduction on CFU/ml of *C. albicans* biofilms as described above. We also tested another phenothiazinium dye NMB (100  $\mu$ M) combined or not with KI (100 mM) with four doses of red laser (10, 20, 40 and 60 J). When NMB was combined with KI the highest log reduction was 1.38 with 60 J of light energy, but the best log reduction was found with this PS without any added KI (1.77 log<sub>10</sub>) and with 60 J of irradiation as well. This group (NMB + 60 J+) was also chosen to use in the *in vivo* study, which showed promising results. A possible explanation for the observation that KI potentiated PDT with MB but not with NMB lies with consideration of the photochemical mechanisms carried out by the two dyes. The production of iodine radicals from iodide anion represents a one-electron transfer reaction, and it is possible that MB carries out more electron transfer reactions (Type I photochemical mechanism), while NMB may carry out more singlet oxygen generation (Type II photochemical mechanism). This hypothesis would require further investigation to confirm it.

Khademi et al. [35] induced oral candidiasis, using *C. albicans* isolated from an azole-resistant patient, with a similar protocol to ours and at 2 days after the last immunosuppression injection, PDT was carried out on the tongue dorsum by use of different concentrations of MB or poly-L-lysine-chlorine (e6) conjugate (pL-ce6) as PSs, followed by a 10 min diode laser illumination at 660 nm ( $n = 6$  each). The most effective PS found in the animals treated with PDT mediated by MB at a concentration of 450 mg/L with reduction from 3.43 log<sub>10</sub> to 0 (CFU/ml) and it was the highest *C. albicans* eradication recorded among all groups in this study.

Costa et al. [36] and Dovigo et al. [37] also worked with a model of oral candidiasis using PDT treatment post infection. The first study used erythrosine (400  $\mu$ mol/L) followed by exposure to a green LED (14.34 J cm<sup>-2</sup>) and the quantified (CFU/ml) yeasts recovered from the mice were significantly reduced by 0.73 log<sub>10</sub>. This group also analyzed the tongues microscopically and found the presence of yeasts and hyphae that were limited to the keratinized layer on the tongue dorsum, which was primarily localized on simple conic papillae. Several lesions in the epithelial tissue were also observed and in the areas with tissue lesions, the lamina propria exhibited a mild inflammatory infiltrate. The groups that received PDT demonstrated a reduction of yeasts, hyphae, lesions and inflammatory infiltrate. The second study used curcumin (20, 40 and 80  $\mu$ M) and illumination with LED light and all animals exposed to curcumin with LED light exhibited a significant reduction in *C. albicans* viability after PDT, but the use of 80  $\mu$ M curcumin and light was able to induce the highest log<sub>10</sub> reduction in colony counts (4 logs) and no evidence of fungi was found on histological examination of the tongues in four animals treated with PDT, with 80  $\mu$ M curcumin.

In contrast to the last 3 studies cited, the aim of our study was to evaluate PDT of oral candidiasis using real-time bioluminescence monitoring. The use of a luciferase-expressing strain of *C. albicans* allowed this real-time monitoring using bioluminescence imaging with an IVIS® Lumina Series III. In our study we found that 5 days of daily PDT treatment with MB (1 mM) combined with KI (1 M) and 40 J of light irradiation and NMB with 60 J of light energy almost eradicated the infection (Fig. 7C and D). On day 1 of treatment only the group NMB + 60 J+ was significantly reduced in comparison with the control group ( $p = 0.012$ ). On day 2 however both PDT groups were significantly reduced,  $p = 0.006$  for NMB and  $p < 0.001$  for MB. From the third day both PSs had  $p < 0.001$  when the treatment with these PSs was compared with the control group. During the 5 days of treatment no difference was found between the treatments with MB and NMB ( $p > 0.05$  for all days). Observing Figs. 6 and 7D we can see that the treatment with MB plus KI was the best one among the four treatments tested in this study, and this combination with 40 J of irradiation was chosen for the animal experimental group.

Under optical microscopy, many yeasts and hyphae, epithelial lesions such as epithelial hyperplasia, basal cell layer disorganization, exocytosis, loss of filiform papillae, hyperkeratosis and intraepithelial micro abscess development, and mild inflammatory infiltrate with a

predominance of neutrophils were observed on the tongue dorsum of the animals without treatment with MB or NMB (Fig. 8A and D). In some cases, moderate inflammatory infiltrate and mononuclear cells in the lamina propria and basal layer were observed, and congested vessels were occasionally noted. On the tongues of the animals that received PDT treatment, we found only occasional yeasts, hyphae, lesions and inflammatory infiltrate. Most of the tongues of the animals that were treated were uninjured (Fig. 8B, C, E and F) demonstrating that PDT with MB (1 mM) combined with KI (1 M) and 40 J of light irradiation and NMB with 60 J of light energy is effective.

Judging by the results obtained in the present study, both NMB-PDT and MB-PDT combined with KI represents two potential antifungal strategies to treat oral candidiasis in an immunosuppressed murine model. A single application of PDT was sufficient to produce elimination of *C. albicans* from some of the evaluated mice (NMB + 60 J+) but the most promising results were observed with the use of 5 days of daily treatment using either PS, suggesting that PDT using our protocols is a promising approach for treatment of oral candidiasis. Considering the non-toxic nature of KI (FDA approved and generally available as a health supplement to avoid iodine deficiency) clinical trials studies using PDT combined with KI could be undertaken to translate our results into clinical practice.

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