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Photodynamic therapy has antifungal effect and reduces inflammatory signals in *Candida albicans*-induced murine vaginitis

R.M. Machado-de-Sena[a,b], L. Corrêa[c], I.T. Kato[a], R.A. Prates[d], A.M. Senna[e,f], C.C. Santos[a], D.A. Picanço[a], M.S. Ribeiro PhD[a,*]

[a] *Center for Lasers and Applications, IPEN-CNEN/SP, Av. Lineu Prestes, 2242, 05508-000 São Paulo, SP, Brazil*
[b] *Department of Microbiology and Immunology, Federal Institute of Education, Science and Technology, IFTO, Av. Amazonas, Qd 56 — Lt 01, 77826-170 Araguaina, TO, Brazil*
[c] *Department of Stomatology, Dentistry School, University of São Paulo, FOUSP, Av. Lineu Prestes, 2227, 05508-900 São Paulo, SP, Brazil*
[d] *Postgraduate Program in Biophotonics Applied to Health Sciences, Nove de Julho University, UNINOVE, Rua Vergueiro, 235, 01504-001 São Paulo, SP, Brazil*
[e] *Department of Oral Surgery, Palmas General Hospital, Av. 201 SUL NS1, Conj. 02 — Lt 01, 77015-202 Palmas, TO, Brazil*
[f] *Department of Lasers and Photomedicine, Tocantinense Institute President Antônio Carlos, Rua 02 Qd 07 s/n, 77500-000 Porto Nacional, TO, Brazil*

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Summary
Background: Vaginal candidiasis (VC) is a disease that affects thousands of women of child-bearing age, mainly caused by *Candida albicans* fungus. Photodynamic therapy (PDT) uses photosensitizing substances that are nontoxic in the dark, but able to produce reactive oxygen species when they are subjected to a light source. In this work our purpose was to investigate PDT effects on fungal burden and inflammatory cells in a murine model of *C. albicans*-induced vaginal candidiasis.
Methods: Female BALB/c mice 6–10 weeks were estrogenized and maintained in this state during all experiment. After 72 h, mices were inoculated intravaginally (IV) with 20 μL of 2×10^5 *C. albicans* cells suspension. Mice were separated into 5 groups after five days: H (healthy), PBS (control), laser, MB (methylene blue) and PDT. PDT and MB groups received IV 20 μL solution with 1 mM of MB, others received PBS. PDT and laser groups were irradiated with a red laser (100 mW, 660 nm) in one (36 J, 6 min) or two sessions (18 J, 3 min). After the end of treatment, mice were submitted to microbiological and histomorphometric analysis with ImageJ software. Data were

* Corresponding author. Tel.: +55 11 31339197; fax: +55 11 31339374.
E-mail addresses: marthasr@usp.br, martharibeiro@yahoo.com (M.S. Ribeiro).

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plotted by mean values and standard deviations of CFU/mL and percentage of inflammatory cells area. ANOVA and Bonferroni post-test were used and data were considered significant when $p < 0.05$.
Results: PDT significantly reduced *C. albicans* after the two tested protocols, however, percentage area of inflammatory cells was significantly reduced just with two sessions of PDT.
Conclusions: PDT with MB and red laser is a promising therapy for VC. It is able to reduce fungal infection in biofilm and inflammatory signals associated with VC in a murine model of vaginitis.

Introduction

Vulvovaginal candidiasis (VVC), a disease caused by *Candida* species (*Candida* spp.), affects 75% of women of childbearing age and 5—8% of these suffer from a recurrent form. Only in Brazil, based on 2010 population census, estimated population growth and data from the National Health System (SUS), the female population of fertile age (10—49 years) is about 64 million.

The primarily etiological agent of VVC is the fungal *Candida albicans*. In most individuals, *C. albicans* remains in the human body as a harmless commensal organism, but once host environment changes, it can become a pathogenic species and affect corporal surfaces causing localized infections, as vaginal candidiasis (VC). This disease is characterized by fungal presence associated with inflammation on the vaginal tissue. In this sense, the VVC can be defined as the presence of signs and symptoms of inflammation on the vagina and vulva in the presence of *Candida* spp., and in the absence of other infectious etiologies [1].

Several studies indicate that the most prevalent species causing VC is *C. albicans*, followed by *C. glabrata* [2—4], and these yeasts are showing resistance to antifungals including fluconazole, a drug that is widely used in the treatment of VVC [5]. Available therapeutic resources are relatively few, especially for species that show antifungal resistance [6].

Fungal vaginitis due to *Candida* spp. cause many symptoms, which can include itching, pain, changes in vaginal secretion, dyspareunia, vulvar erythema, edema and fissures [7]. Such vaginitis can be classified into complicated and uncomplicated. Among the complicated VVC, a recurrent form is characterized when four or more episodes of the illness occur over a period of 12 months and may be caused by *C. albicans* and non-*albicans* species, however, the treatment in both cases is more difficult [8].

A treatment possibility appears with photodynamic therapy (PDT), which uses a photosensitizer (PS) dye that activated by light induces the formation of reactive oxygen species (ROS), causing death of microorganisms by oxidative damage [9]. Besides, this therapy has minimal prejudicial effects on human cells, but is selectively toxic for microorganisms [10].

In vitro studies have shown that dyes such as methylene blue (MB) can be activated by light to produce antimicrobial effects against diverse types of fungus [11—13]. *In vivo* studies, although scarce, have also been carried out demonstrating the antimicrobial effect of MB activated by light. For example, rodents such as mice and rats were used in models for the treatment of induced fungal and bacterial infections and also for wound healing [14—17]. Although rare, volunteers' human studies have begun with good results in the treatment of fungal infection on oral mucosa [18].

New therapeutic approaches for the treatment of VVC are need and due to scarcity of *in vivo* studies of PDT on vaginitis, we developed this trial to investigate PDT effects on fungal burden and inflammatory cells in a murine model of *C. albicans*-induced vaginal candidiasis using MB as photosensitizer and a red laser as the light source.

Methods

Laboratory animals

All animal experiments were approved by the Committee of Ethics on animal research of IPEN-CNEN/SP. Seventy seven female BALB/c mice aging 6—10 weeks, supplied by the Central Animal House of IPEN, were selected for this study (Fig. 1). Animals were housed in individual cages with water and food *ad libitum* and were kept in a 12:12 h light/dark cycle in a controlled temperature ($22 \pm 2\,^{\circ}C$). All experiments were designed to minimize animal suffering and the procedures were performed under general anesthesia by intraperitoneal injection of ketamine ($90\,mg\,kg^{-1}$) and xylazine ($10\,mg\,kg^{-1}$).

Strain and inoculum preparation

C. albicans (ATCC 90028) was cultured overnight on Sabouraud dextrose agar (SDA) at 37 °C. Cells were collected, suspended in phosphate buffered saline (PBS) and homogenized in a vortex shaker. The concentration was estimated by the turbidity of the suspension, which was measured by spectrophotometer at 540 nm. Suspensions were prepared with an optical density of 0.8, corresponding to the concentration of about 10^7 CFU/mL (colony forming units per milliliter) [19]. Inoculum concentration was confirmed by cultivation on SDA and counting CFU/mL, according to the methodology proposed by Jett et al. [20].

Induced vaginal candidiasis

In order to induce a pseudo-estrus condition, mice received weekly subcutaneous injection of 0.1 mg of 17-β estradiol valerate (Sigma®) dissolved in 0.1 mL of sesame oil (Lipo do Brasil®) 72 h prior to *C. albicans* inoculation throughout the experimental period [21]. Infection was induced by intra-vaginally inoculation (IV), of 20 μL of a 10^7 cells/mL of

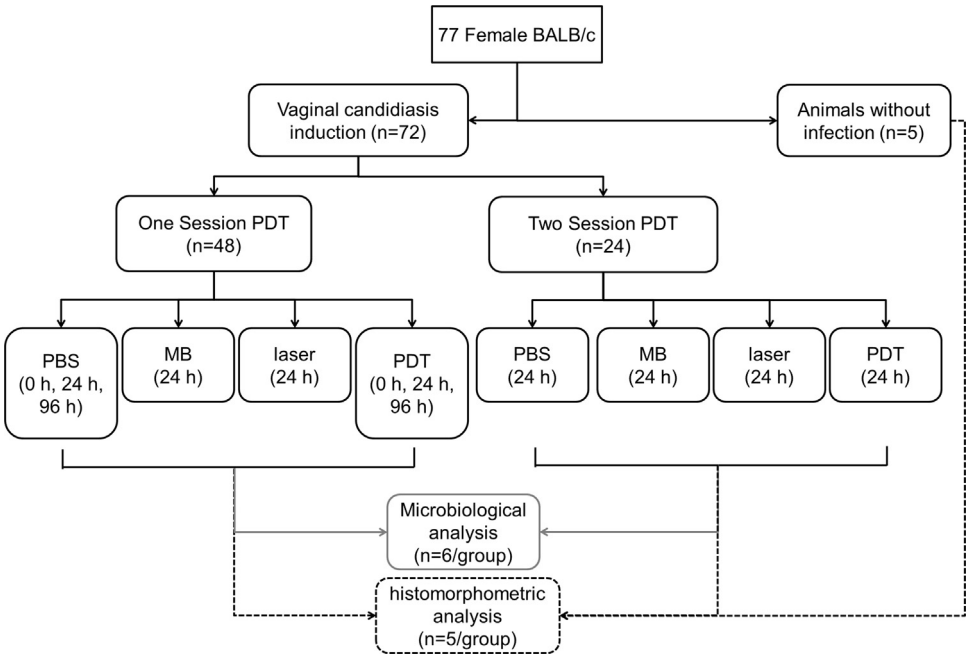

Figure 1 Flowchart diagram of the study design. Animals were evaluated immediately (0 h), 24 h or 96 h after treatment.

C. albicans suspension. Fig. 2 summarizes the experimental procedure.

Light source, photosensitizer and treatments

In this *in vivo* study, two different protocols of PDT were used and treatments were performed five days after the induction of *C. albicans* infection. In each protocol, mice were divided into four groups: PBS, MB, laser and PDT (Fig. 1). In PBS and laser groups, animals were inoculated with 20 μL of sterile PBS, and in MB and PDT, with 20 μL of 1 mM MB solution. PBS and MB groups did not receive light, while PDT and laser groups were irradiated with red laser (Photon Lase III DMC, Brazil, calibrated with Laser Power Meter Check mark — Coherent®, USA) in one session (λ = 660 nm, 100 mW, 36 J, 6 min) or two sessions with a 24 h interval (λ = 660 nm, 100 mW, 18 J, 3 min). The laser tip (Φ = 2 mm) was carefully placed on the entrance of the vaginal canal. Irradiation was conducted 10 min after MB inoculation. Animals were evaluated immediately (0 h), 24 h or 96 h after treatment, according to description presented in Fig. 1. For each moment of the study, different animals were used. Besides these animals, 5 healthy mice (H) were monitored as negative control of the disease.

Microbiological analysis

Vaginal lavage was done by repeated wash and aspiration of vaginal cavity using 50 μL of PBS [22]. The lavage fluids were serially diluted and cultured on SDA with chloramphenicol at 37 °C for 28 h to determine CFU/mL. These events were

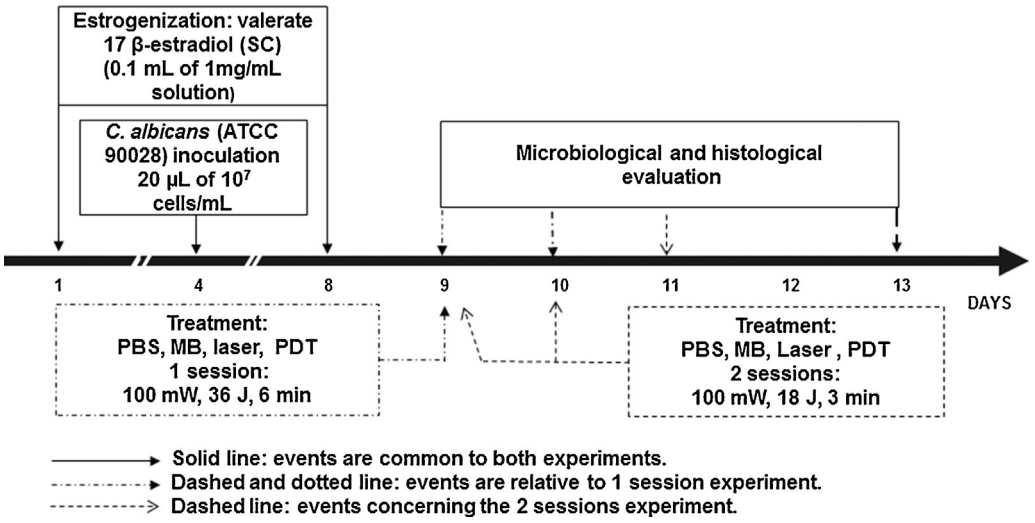

Figure 2 Timeline of experimental procedure.

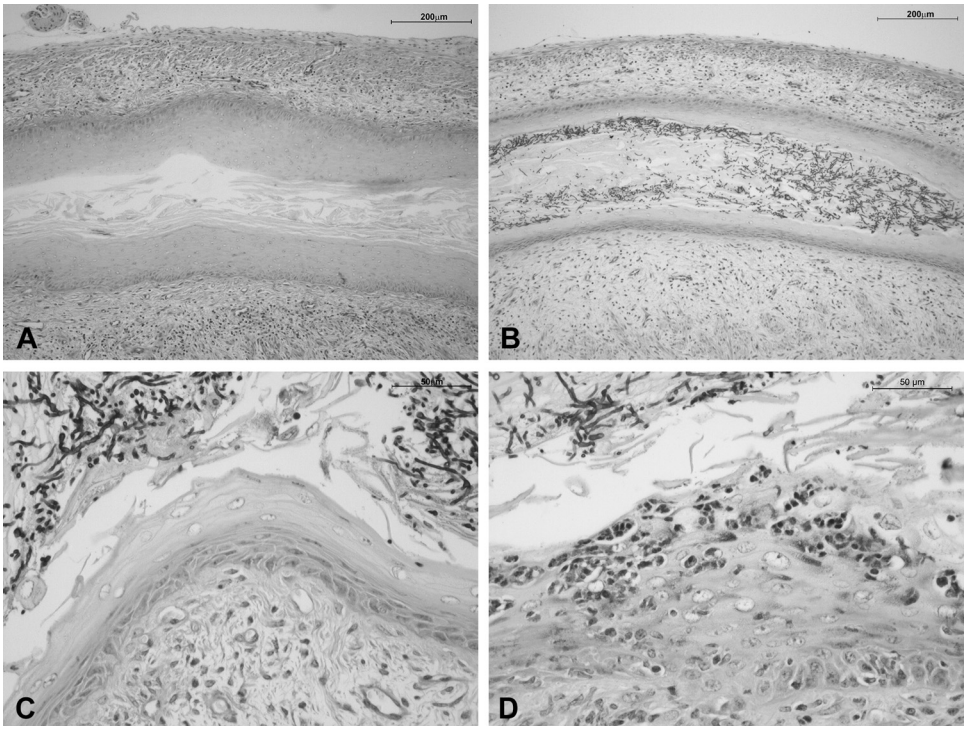

Figure 3 Photomicrographies of vaginal canal of mice stained by PAS. (A) Healthy animal showing the stage of estrus, characterized by keratinized stratified squamous epithelium with several layers and intense keratinization. The lamina propria shows large amount of fibroblast cells and some inflammatory mono and polymorphonuclear cells; (B) PBS group revealed a large number of suggestive *Candida* fungus in lumen of the vaginal canal; at higher magnification, note epithelial atrophy and inflammatory infiltrate of mono and polymorphonuclear cells diffused in lamina propria (C) and presence of microabscess and exocytosis by polymorphonuclear cells in the epithelial superficial layers (D).

performed immediately (0 h), 24 h or 96 h after treatment (Fig. 2).

Morphologic and histomorphometric analysis

Following vaginal lavage, mice were euthanized in a CO_2 chamber, vaginal canals were removed and immediately fixed in 10% (v/v) buffered formalin. Longitudinal sections were made and the samples were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). PAS is a staining method to detect polysaccharides and mucosubstances in tissues that is used to mark cell walls at living fungi.

Inflammatory response related to the presence of *C. albicans* was evaluated by measuring the area of nucleus of inflammatory cells present in vaginal tissue. To this aim, slides containing longitudinal section were stained with HE and 12 samples of the vaginal canal were taken. Vaginal tissues from all groups were analyzed, including healthy mice. All analyses were performed in a blinded fashion by a single examiner.

Inflammatory cells present on vaginal connective tissue were photographed and the images were analyzed by ImageJ software as a percentage area of nucleus of inflammatory cells [23,24].

Data analysis and statistics

Values were presented as means and standard deviations of CFU/mL *of C. albicans* and percentage area of nucleus of inflammatory cells. The treatment groups were compared for statistical significance, using Anova One Way and Bonferroni post-test. The value of $p < 0.05$ was considered significant.

Results

The behavior of infected animals was not changed after induction of vaginitis, and there were no changes in the amount or volume of feces and urine. Also no movement on the limbs or body of females that could demonstrate the existence of vulvovaginal pruritus was perceived. However, some characteristic signs of human vaginitis were found in some animals such as edema, erythema and discharge in the genital area.

Model establishment

As can be observed from Fig. 3, vaginal tissue of healthy animals exhibited a large amount of fibroblasts and small number of inflammatory cells (Fig. 3A). Histological analysis of infected mucosa before treatment (day 9) showed epithelial damage, blastoconidia and hyphae of *C. albicans* in the keratinized layer of the epithelium and in the vaginal lumen (Fig. 3B).

We successfully developed a murine model of VC since stage of estrus could be confirmed by a stratified and intensely keratinized epithelium. We did not observe any

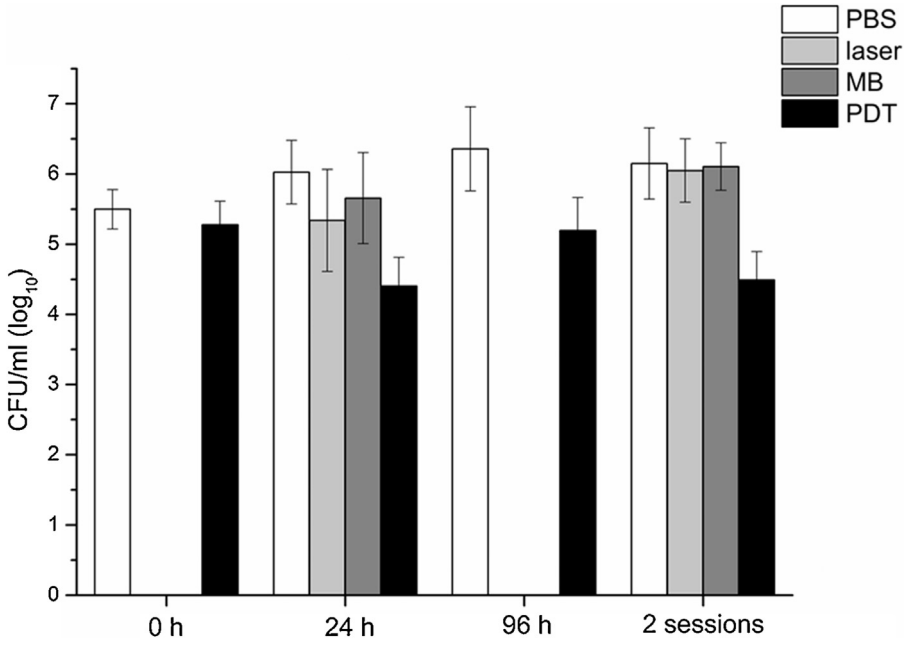

Figure 4 Mean values ± SD of vaginal fungal burden in candidiasis induced in BALB/c mice. Results from 0, 24 and 96 h correspond to one session of PDT. 2x represent data obtained 24 h after treatment with two sessions of PDT.

fungus on the vaginal canal lumen (Fig. 3A). Only vaginal tissue infected by *C. albicans* presented an inflammatory response (Fig. 3B). We noticed a large inflammatory infiltration diffused through the lamina propria (Fig. 3C) and intraepithelial microabscesses consisting of polymorphonuclear cells (Fig. 3D).

Antifungal effects of one session of PDT

PDT was applied in infected mice and the fungal burden was monitored at 0, 24 and 96 h after the end of treatment. During the experimental period, the untreated PBS group showed no statistically significant increase on the fungal load (Fig. 4). Regarding the results of PDT group, immediately after treatment (0 h), the fungal burden (5.28 log) showed no difference compared to PBS group. After 24 and 96 h, the PDT group showed a reduction of 1.62 log CFU/mL (24 h, $p < 0.05$) and 1.16 log (96 h, $p < 0.05$) compared to PBS. In addition, fungal killing was significantly higher at 24 than 96 h after PDT ($p < 0.05$). MB and laser groups showed no significant reduction in *C. albicans* counts compared to the PBS group.

Antifungal effects of two sessions of PDT

In this part of the study, the energy of 36 J used in the previous experiment was fractionated into two sessions with 18 J of energy, respecting an interval of 24 h between irradiations.

Our results showed that this PDT protocol promoted a fungal reduction of about 1.66 log CFU/mL compared to PBS ($6.15 \pm 0.45 \times 4.49 \pm 0.41$, $p < 0.05$). MB and laser groups showed no significant reduction in microbial count when compared to PBS group (Fig. 4).

MB-mediated PDT showed antifungal activity in induced *C. albicans* vaginitis model when it was applied in one session with 36 J, as in 2 sessions with 18 J. No differences were found in the antifungal effects between the tested protocols ($p > 0.05$).

Effects of PDT on inflammation of vaginal tissue

Quantitative analysis was performed to evaluate the percentage nucleus area of inflammatory cells. Our results show that *C. albicans* infection increased the area of nucleus of inflammatory cells when compared to healthy animals ($p < 0.05$). The data demonstrate that the area in the PBS group significantly increased from 0 to 24 h ($p < 0.05$) and then remained constant up to 96 h.

All animals infected with *C. albicans*, treated or not with PDT, presented a higher area of inflammatory cells when compared to healthy animals. PBS, MB, laser and PDT applied in one session did not alter the area of inflammatory cells in the vaginal canal of infected animals. On the other hand, interestingly, PDT performed in two cycles showed a significant reduction of percentage area of inflammatory cells ($p < 0.05$); although this reduction did not reach normal values found in healthy animals (Fig. 5).

Fig. 6 substantiates our findings. As can be observed, healthy animals exhibited fibroblast cells and some inflammatory cells in lamina propria of vaginal canal mucosa. Contrarily, PBS control group shows intense inflammatory infiltrate, predominantly polymorphonuclear cells. Mice of PDT group applied in two sessions displayed discrete inflammatory infiltration in the lamina propria 24 h after second session.

Discussion

The vaginitis, including candidiasis, is still a problem for public health and it is among the most common reasons for women seeking gynecological care. VVC is responsible

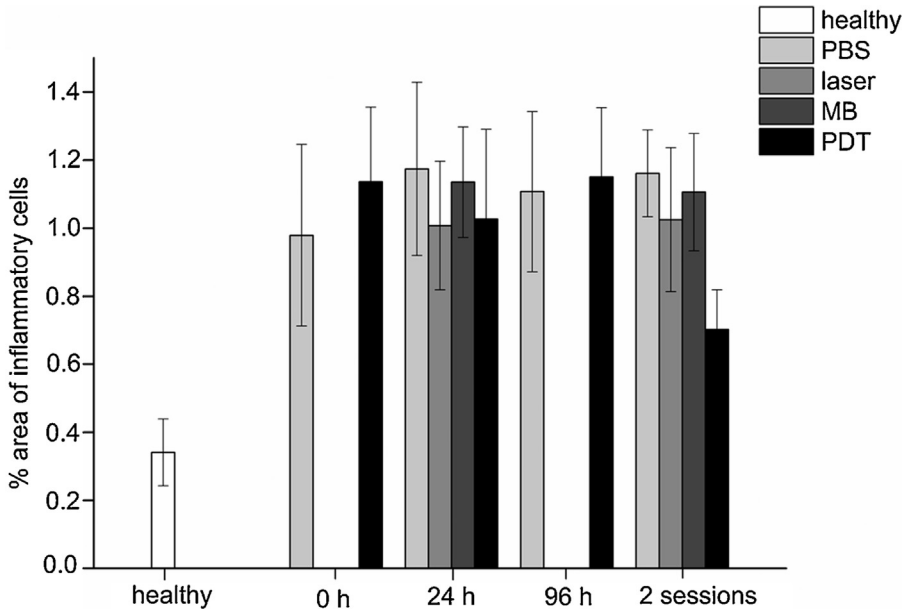

Figure 5 Mean values ± SD of percentage area of inflammatory cells on *C. albicans*-induced vaginitis in BALB/c mice. Results from 0, 24 and 96 h correspond to one session of PDT. 2x represent data obtained 24 h after treatment with two sessions of PDT.

for about 13 million vaginitis in North American women each year. In Brazil, 64 million women of childbearing age are susceptible to VVC [25]. The search for more effective therapies has been frequent, which encourages the study of alternatives to existing treatments.

In this work, we investigated the use of PDT as a strategy to treat vaginal candidiasis. Besides the reduction of fungal burden, we also aimed to decrease tissue inflammation, since vaginal candidiasis is characterized by an inflammatory response to *Candida* rather than solely fungal colonization [8]. That being the scope of this study, we first established a model of vaginal candidiasis in female BALB/c mice.

Similar to women, *C. albicans* can colonize mice vaginal tissue without promoting disease [21]. Notwithstanding this challenge, we employed a methodology to induce candidiasis that resulted in a high fungal burden, the presence of hyphae forms and microabscesses, and an increase of inflammatory cells in vaginal tissue. These signs strongly indicate a disease phenotype [21,26], similar to symptomatic infection experienced by women with VVC.

PDT has been used successfully to treat different localized infections [10,15—18]. Our results demonstrate that PDT is also a suitable method to promote *C. albicans* reduction and diminish inflammatory status of vaginal tissue. Among the existing works on fungal infection, PDT is generally applied in models of oral candidiasis [14,27]. However, studies of PDT effects on *C. albicans* vaginitis are currently lacking in literature.

Teichert and collaborators used red laser and MB on murine oral mucosa and demonstrated that total reduction in fungal burden was dependent on photosensitizer concentration and light parameters [14]. Junqueira and co-workers [27] also treated oral candidiasis in rats, but the results showed no decrease in fungal burden, although treated rats presented fewer injuries due to candidiasis than those untreated. It should be remarked that the study design and the protocol used in the two studies were different, starting with the immune status of the animals. In the first case, animals were immunosuppressed, whereas the latter were normal.

Our results showed that treatments made only with laser or MB showed no antifungal activity, in agreement with literature [11,14,18]. Following one or two sessions of PDT, we achieved about 1.6 log in fungal reduction 24 h after

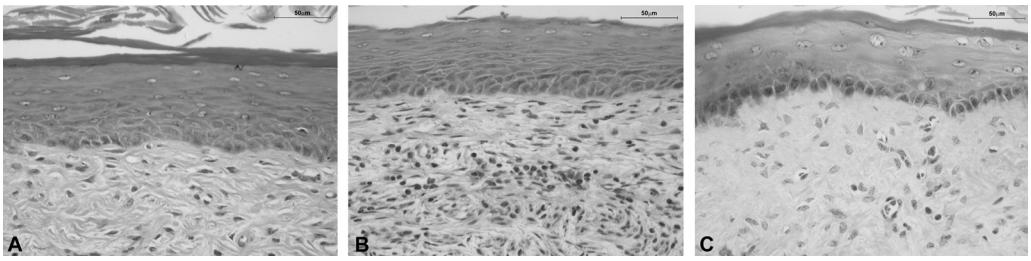

Figure 6 Photomicrographies of vaginal mucosa of mice stained by HE. (A) Healthy animal, showing lamina propria exhibiting fibroblast cells and some inflammatory cells; (B) PBS group shows intense inflammatory infiltrate, predominantly polymorphonuclear cells diffused in the lamina propria; (C): PDT 2 sessions group presents discrete inflammatory infiltrate in the lamina propria.

treatment. These data corroborate studies that investigated effects of PDT in biofilm or animal models using phenotiazines and red light on *C. albicans* [28,29]. In fact, it is well known that microorganisms' susceptibility to PDT decreases when they are organized in biofilms.

We also observed that a significant *C. albicans* reduction was maintained after 96 h compared to control group, but, at this point, a fungal recolonization tends to occur since fungal load was significantly increased when compared to 24 h. This finding fits with Prates et al. who showed a significant recolonization and/or regrowth of microaerophile bacteria 7 days after PDT in their model of mice induced-periodontitis [15]. Thus, when we decided to split energy in two cycles of PDT we chose evaluate *C. albicans* fungal and inflammatory infiltration only 24 h after the second PDT session. We assumed that antifungal effect of PDT would be more pronounced if *C. albicans* virulence was diminished [30].

On the other hand, interestingly, inflammatory cells were reduced only after two PDT cycles (18 J, 3 min and 24 h interval). With this protocol, PDT was able to decrease the percentage area of inflammatory cells caused by *C. albicans* and it may be due to the decrease of virulence on the remaining yeast after the first PDT session. According to Kato et al. [30], sublethal doses of photodynamic therapy can reduce fungal virulence by decreasing the formation of germ tubes (GT), which is an important factor in the pathogenicity of *C. albicans*. In fact, Sobel et al. [31] demonstrated that the germ tube formation plays a critical role in the pathogenesis of vaginitis caused by *C. albicans*, since the absence of the tube reduces the adhesion of fungal on the vaginal mucosa. Additionally, PDT can inactivate microorganisms and their enzymes and this fact decreases harmful effects of toxins on vaginal mucosa.

A limitation of the vaginal candidiasis model used in this study is the fact that it is not possible to follow the infection in the same female. The method used, in principle, allows no more than one wash in the same vaginal tissue. This requires a large number of animals for longer studies, which requires time, cost and involves important ethical issues.

This work is a pioneer in understanding the effects of PDT induced in *C. albicans* vaginitis. It shows that it is possible to reduce the fungal infection and its associated inflammatory signs. This fact is very important for extrapolating this therapy to humans, since the symptoms of the disease in women is mainly due to inflammation caused by the fungus rather than the infection itself. However, more studies are needed to use this therapy in the treatment of fungal vaginitis or of another etiology in animal models as well as for its use in humans.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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