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Original Communication

Effects of helium-neon (HeNe) laser treatment on *Paracoccidioides brasiliensis* yeast cells

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ABSTRACT

In this work, we evaluated the effects of laser treatment on fungal cells, aiming to provide support for the use of this therapy during Paracoccidioidomycosis (PCM). HeNe laser treatment reduced the growth capacity and the viability of fungal cells, and increased gp43 expression. Laser-treated cells also showed pronounced structural alterations, since they were collapsed and presented deep folds. Additionally, remarkable changes were observed in the nucleus that presented a very electron dense form. The pathogenicity of *P. brasiliensis* after laser treatment was also evaluated, and no differences were observed in pulmonary lesions, comparing animals infected with laser-treated and non-treated yeast cells. Possibly HeNe laser is able to modify fungal structures involved in resistance and virulence mechanisms and therefore can be considered as an adjunctive therapy in the treatment of skin lesions observed during PCM.

KEYWORDS: low level laser therapy, HeNe laser, *Paracoccidioides brasiliensis*, yeast cells

INTRODUCTION

The human pathogenic fungus *Paracoccidioides brasiliensis* (*P. brasiliensis*) is the etiological agent of Paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in several Latin American countries [1]. This pathogenic dimorphic fungus grows in a mycelial form at environmental temperatures (20-26°C), and in a yeast phase at the temperature of the mammalian host (36-37°C). The disease is acquired by inhalation of dust containing the infecting propagules produced by the fungal mycelium. The infection takes place first in the lungs and afterwards is disseminated via the circulatory and/or lymphatic system to different organs such as the liver, spleen, adrenal glands, skin and mucosae, among others, where it induces severe lesions and remains viable even with antimycotic therapy [2].

The cutaneous lesions, which are extremely painful and sensitive, can progress to ulcerative wounds and predispose the patients to secondary bacterial infections [3, 4]. The currently available treatment with antifungal agents is frequently toxic at therapeutic dosages, and some drugs affect structures that are shared by humans and yeast causing side effects to patients.

Because of the discomfort caused by lesions and collateral drug effects, new non-harmful strategies are being proposed that can be used as adjuvant and/or alternative therapy to eradicate the pathogen and accelerate the cure of lesions. In this way, low intensity laser therapy using helium-neon (HeNe) laser irradiation can represent an excellent tool

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due to its microbicidal and anti-inflammatory effects, wound healing properties, and non-invasive characteristics that are already well known [5, 6, 7].

Previous studies from our laboratory, which explored the biological mechanisms underlying the inflammatory process of paracoccidioidomycotic lesions treated by HeNe laser, demonstrated the ability of this therapy to increase the production of proinflammatory cytokines as well as the synthesis of extracellular matrix components, leading to faster and more effective wound healing. In addition, fungal cells recovered from the lesions after laser treatment showed no capability of growth when cultured *in vitro* [8, 9, 10].

The present study evaluates the effects of HeNe laser treatment on *P. brasiliensis* yeast cells, aiming to provide additional support for the use of this therapy as an adjuvant tool to be combined with anti-fungal agents in the treatment of PCM ulcerations.

MATERIAL AND METHODS

Fungus

The virulent isolate Pb 18 of *P. brasiliensis* was maintained in the yeast form at 37ºC in Fava Netto's culture medium, and was used at the 7^{th} day of cell culture growth.

Laser treatment

P. brasiliensis isolate (Pb 18) in its yeast phase was grown in Petri dishes containing semi-solid BHI medium supplemented with 4% (vol/vol) fetal calf serum and 5% (vol/vol) yeast extract of *P. brasiliensis*, and incubated at 36ºC. After seven days in culture, *P. brasiliensis* colonies were individually exposed to HeNe laser (incident energy of 3 J/cm^2) for 110 seconds for three consecutive days. Twenty-four hours after the last exposure, *P. brasiliensis* cells were collected from the cultures; suspensions were prepared and cells were analyzed for growth inhibition, viability, and infectivity. For ultra-structural analyses, *P. brasiliensis* yeast cells were evaluated after 2 and 24 hours of laser treatment. Non-treated cultures were maintained in parallel and used as control.

Yeast cell viability by Trypan Blue

HeNe laser-treated fungal cultures were resuspended in PBS 0.15 M pH 7.4 and the concentration was adjusted to 1 x 10^6 cells/mL. 50 µL of the suspension were incubated with 50 µL of Trypan Blue and dead cells were tallied in a Neubauer chamber.

Growth inhibition

Individual colonies were collected and resuspended in 1 ml PBS, washed and counted in a Neubauer chamber. Inoculum concentrations ranging from 10^3 to 10^7 cells/mL were plated in Petri dishes, incubated for 7 days at 36°C, and colony forming units (CFUs) were counted.

SEM

The morphological alteration of *P. brasiliensis* yeast cells were monitored by scanning electron microscopy (SEM). Laser-treated and non-treated *P. brasiliensis* yeast cells were washed in PBS 0.1 M pH 7.4 and fixed for 12 h in PBS 0.1 M pH 7.4 solution containing 2% of paraformaldehyde and 1.5% of glutaraldehyde. The samples were then fragmented and post fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 2 h. After thorough rinsing in 0.1 M phosphate buffer, samples were dehydrated in a graded series of ethanol and transferred into liquid CO_2 in a critical point dryer. The dried specimens were mounted, sputter coated with gold, and examined under the scanning electron microscope JEOL 5800LV (Jeol BV, Zaventem, Belgium).

TEM

The ultra-structural alteration of *P. brasiliensis* yeast cells were surveyed by transmission scanning electron microscopy (TEM). For chemical fixation, immediately after collection, laser-treated and non-treated *P. brasiliensis* yeast cells were washed three times with PBS 0.1 M pH 7.4. Afterwards, samples were fixed overnight in PBS 0.1 M pH 7.4 solution containing 2% of paraformaldehyde and 1.5% of glutaraldehyde. After fixation, they were rinsed in PBS 0.1 M pH 7.4 and fragmented. Postfixation was done in PBS 0.1 M pH 7.4 solution containing osmium tetroxide 1% for 90 min, followed by a series of ethanol dehydration steps. The fragments were

then washed with increasing concentrations of propylene oxide and infiltrated with propylene oxide/Epon (1:1) and pure Epon resin. The sections were contrasted with uranyl acetate and lead citrate, and examined using a LEO 906 transmission electron microscope (ZEISS, Oberkochen, Germany).

Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed to analyze and compare the expression of the secreted fungal cell wall protein, the 43,000-Da glycoprotein (gp43), as previously described [11]. The membranes were incubated with an anti-gp43 polyclonal antibody (kindly provided by Prof. RoneiMamoni from University of Campinas) for 1 h at room temperature. The membranes were washed in phosphate-buffered saline-Tween 20 (PBS-T) and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG secondary antibody (Dako) in PBS-T for 1 h at 37ºC. The sites of antibody-antigen reactions were visualized with a solution containing 5 mg of 3,3′diaminobenzidine (Sigma) and 10 µl of H_2O_2 in 50 ml of Tris buffer, pH 7.4.

***In vivo* infection**

Specific pathogen free C57Bl/10 male mice, 6-8 week-old, obtained from Centro Multi-Institucional de Bioterismo (CEMIB) were used. Animals were maintained in transparent acrylic plastic isolators under aseptic conditions throughout the study with sterile water and feed provided *ad libitum*. All animals received humane care in compliance with the guidelines proposed by the Brazilian Council on Animal Care (SBCAL) and the experimental protocol was approved by the animal care and use committee of the University of Campinas (CEUA/UNICAMP). One day after the last laser treatment of *Paracoccidioi desbrasiliensis* cultures, groups of fifteen mice were inoculated intraperitoneally with 5 x 10^6 viable yeast cells/animal. Control groups were also inoculated via i.p. route with 5 x 10^6 non-laser-treated *P. brasiliensis* cells. At days 7, 14 and 30 post infection, five animals from both experimental and control groups were sacrificed by cervical dislocation and lungs removed for histopathological analysis.

Lung histopathology

The lungs were removed surgically and fixed in 4% paraformaldehyde solution for 16 hours at 4ºC. The specimens underwent diafanization with xylene, dehydrated by graded ethanol, and embedded in paraffin. Slices 5-µm thick were cut and stained with haematoxylin-eosin (HE).

Statistical analysis

When required, the Mann Whitney test was employed to determine the significant differences between control values and experimental values. The level of significance was defined as $p \leq 0.05$.

RESULTS

Yeast cell viability by Trypan Blue

The viability of *P. brasiliensis* yeast cells following exposure to HeNe laser irradiation was measured by Trypan blue assay. Figure 1 shows that the viability of laser-treated cells decreased by approximately 30%.

Growth inhibition

To evaluate the effect of laser irradiation on fungi growth, different inoculum concentrations of non-treated and laser-treated yeast cells were plated in Petri dishes, incubated for 7 days at 36°C, and colony forming units (CFUs) were counted. An important reduction of yeast growth was observed in laser-treated cells, reaching approximately 90% when a 10^7 cells/mL inoculum was used (Figure 2).

SEM

Laser-treated yeast cells were examined by scanning and transmission electron microscopy to evaluate the fungal structure after the treatment. Non-treated *P. brasiliensis* yeast cells presented a regular morphology with oval shape and multiple sprouts (Figure 3A). Observations made 2 hours after the first treatment and 24 hours (Figures 3B and 3C, respectively) after the last laser treatment, showed that yeast cells had pronounced structural alterations. Most of the yeast cells were collapsed and presented deep folds.

TEM

The ultra-structural features of non-treated *P. brasiliensis* yeast cells by TEM analysis (Figure 4A) include typical rounded cells with

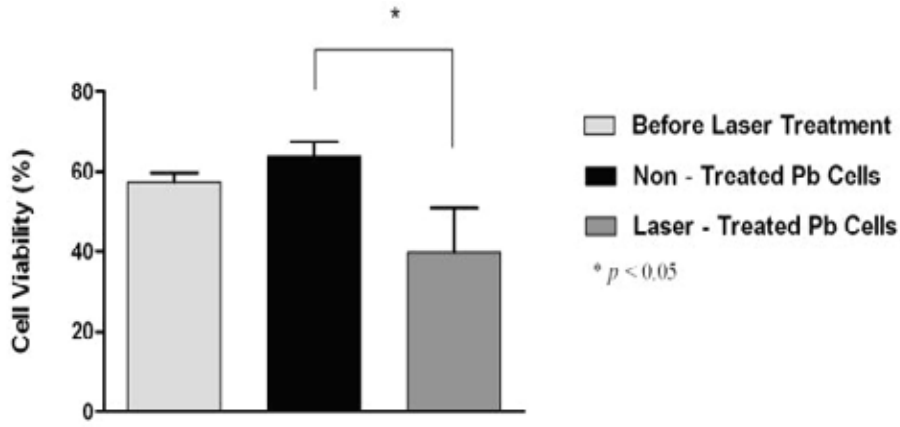

Figure 1. Effect of HeNe laser on *P. brasiliensis* cells viability. Fungus colonies were individually exposed to HeNe laser for three consecutive days, and one day after the last exposure yeast cells were collected from the cultures and analyzed for viability. There was an increase in cell death with HeNe laser exposure compared with control groups. Values are expressed as means ± SE, with n = 6 independent experiments.

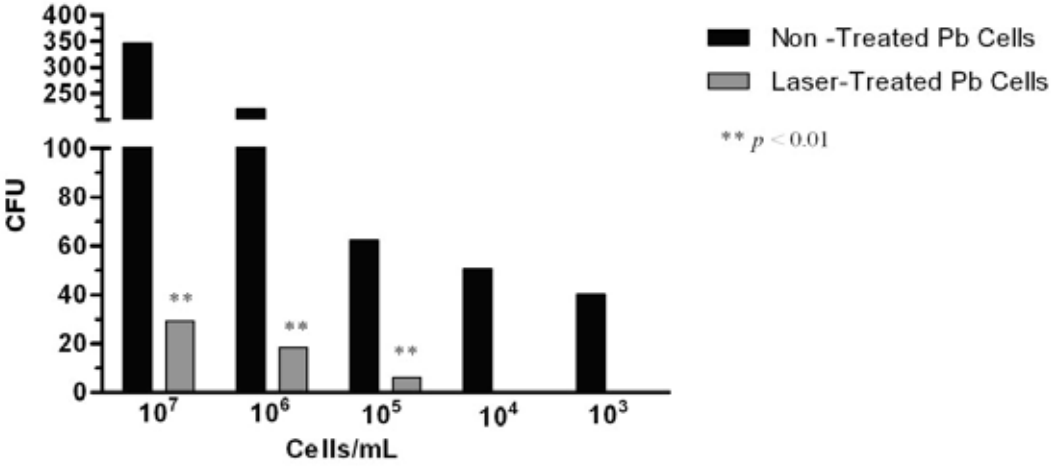

Figure 2. Effect of HeNe laser on *P. brasiliensis* cell growth. Fungus colonies were individually exposed to HeNe laser for three consecutive days, and one day after the last exposure yeast cells were collected from the cultures and analyzed for growth inhibition. A decrease in cell growth was noted after HeNe laser exposure compared with the non-treated group. Values are expressed as means ± SE, with n = 6 independent experiments.

thick walls of low electron density, easily visible plasma membrane, and several vacuoles and mitochondria varying in both shape and size. Laser-treated yeast cells showed both cell wall and plasma membrane intact and preserved (Figure 4B), despite structural changes observed in the SEM analysis. Also, mitochondria and vacuoles were not significantly different from non-treated yeast cells. However, laser-treated yeast cells were more electron dense at the nuclear and cytoplasmic regions and showed less evident cytoplasmic membranes than non-treated ones.

Immunoblotting

The expression of the 43-kDa glycoprotein (gp43), the immunodominant antigen and the main antigenic component of *P. brasiliensis*, was analyzed by immunoblotting reaction obtained

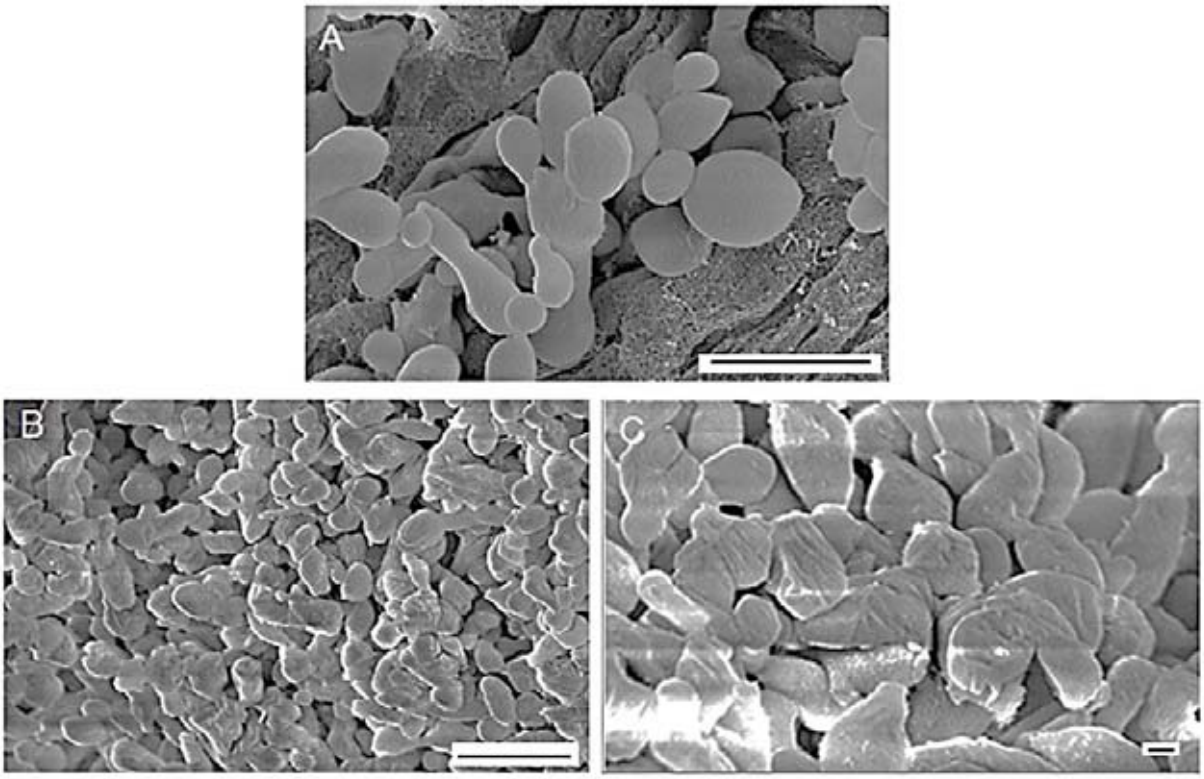

Figure 3. Representative SEM micrographs of non-treated (A) and laser-treated *P. brasiliensis* yeast cells collected two hours after the first treatment (B) and one day (C) after the last treatment. Note that laser-treated yeast cells present deep folds and are collapsed. Results are representative of 6 similar experiments. Bar: 10 µm (A and B); 1 µm (C).

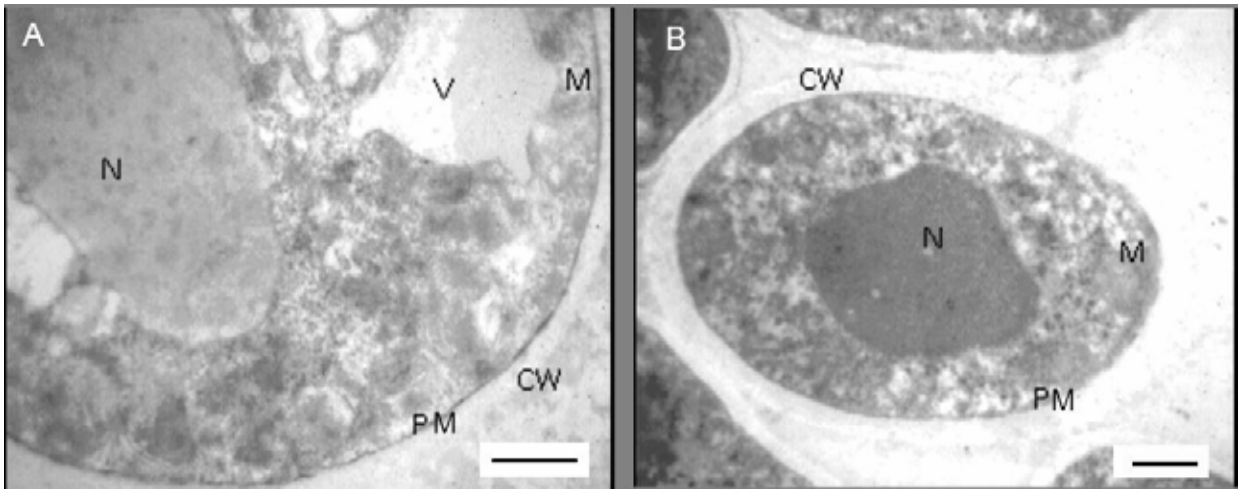

Figure 4. Representative TEM micrographs of non-treated (A) and laser-treated (B) *P. brasiliensis* yeast cells collected 24 hours after the treatment. Note that the laser-treated cell plasma membranes (PM) and cell wall (CW) are intact and preserved, while remarkable changes can be observed in the nucleus (N), which presents a very electron dense form. Mitochondria (M) and vacuoles (V) are not significantly different from non-treated cells. Results are representative of 6 similar experiments. Bar: 10 µm.

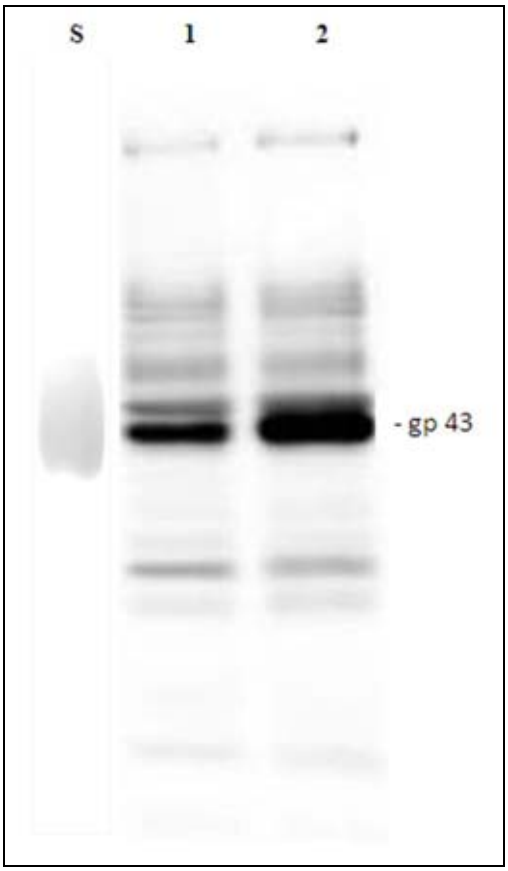

Figure 5. Representative immunobloting reactions obtained with non-treated (lane 1) and laser-treated *P. brasiliensis* cells (lane 2) probed with anti-gp43 polyclonal antibody. Note increase of gp43 expression in laser-treated *P. brasiliensis* yeast cells. S, standard purified gp43.

with crude exoantigen from non-treated and laser-treated yeast cells (Figure 5). Results show an increase of gp43 expression by laser-treated *P. brasiliensis* yeast cells in comparison with non-treated cells. This result was confirmed by ELISA assay (data not shown).

Lung histopathology

In order to evaluate the effects of laser irradiation on fungal pathogenicity, C57BL/10 mice were infected with non-treated and laser-treated *P. brasiliensis* yeast cells. Figure 6 shows the histology of the lesions in lungs after 7, 14 and 30 days of infection with non-treated (left panel) and laser-treated (right panel) yeast cells. At 7 days of infection (Figures 6A and 6B), the main difference between the two groups was the presence of a more intense inflammatory process around blood vessels and newly recruited macrophages in lungs from mice infected with non-treated yeast cells. The presence of numerous yeast cells in clusters, edema, and congestion were also observed. However, at 14 (Figures 6C and 6D) and 30 days (Figures 6E and 6F) after infection no differences were observed in the characteristics of the lesions between animals receiving laser-treated and non-treated yeast cells, since both groups present a similar lung inflammation pattern and multiple granulomatous lesions with many giant cells presenting phagocytized fungal cells. Moreover, at this time of infection no difference in fungal load was observed between lungs from mice infected with non- or laser-treated yeast cells (data not shown).

DISCUSSION

A number of studies have reported positive results for the use of low-intensity laser with therapeutic purposes [12, 13, 14, 15, 16, 17, 18]. Laser therapy for skin lesions caused by *P. brasiliensis* has been studied by our group, showing inflammation reduction of the injured areas, and improvement of tissue healing [8, 9, 10].

Nevertheless, it has been argued that microorganisms might not be affected by the radiation produced by low-power lasers unless they are previously treated with a photosensitizer, such as Methylene blue [19, 20].

The use of different lasers as microbicides has been investigated previously, and the results indicate that they are effective against a variety of microorganisms [21, 22, 23]. Türkün and co-workers [24] have demonstrated antibacterial activity of Er, Cr:YSGG laser with two different power outputs on *S. mutans*. More recently, Yasuda and his group [25] have evaluated the effects of Nd:YAG and Er:YAG lasers on an *Enterococcus faecalis*-infected root canal model. The authors showed that both lasers have a bactericidal effect in infected root canals.

In this work, we investigated the effects of HeNe laser on *P. brasiliensis* yeast cells without using any photosensitizer. Our findings show that laser treatment was very effective in significantly reducing the viability and growth capacity of

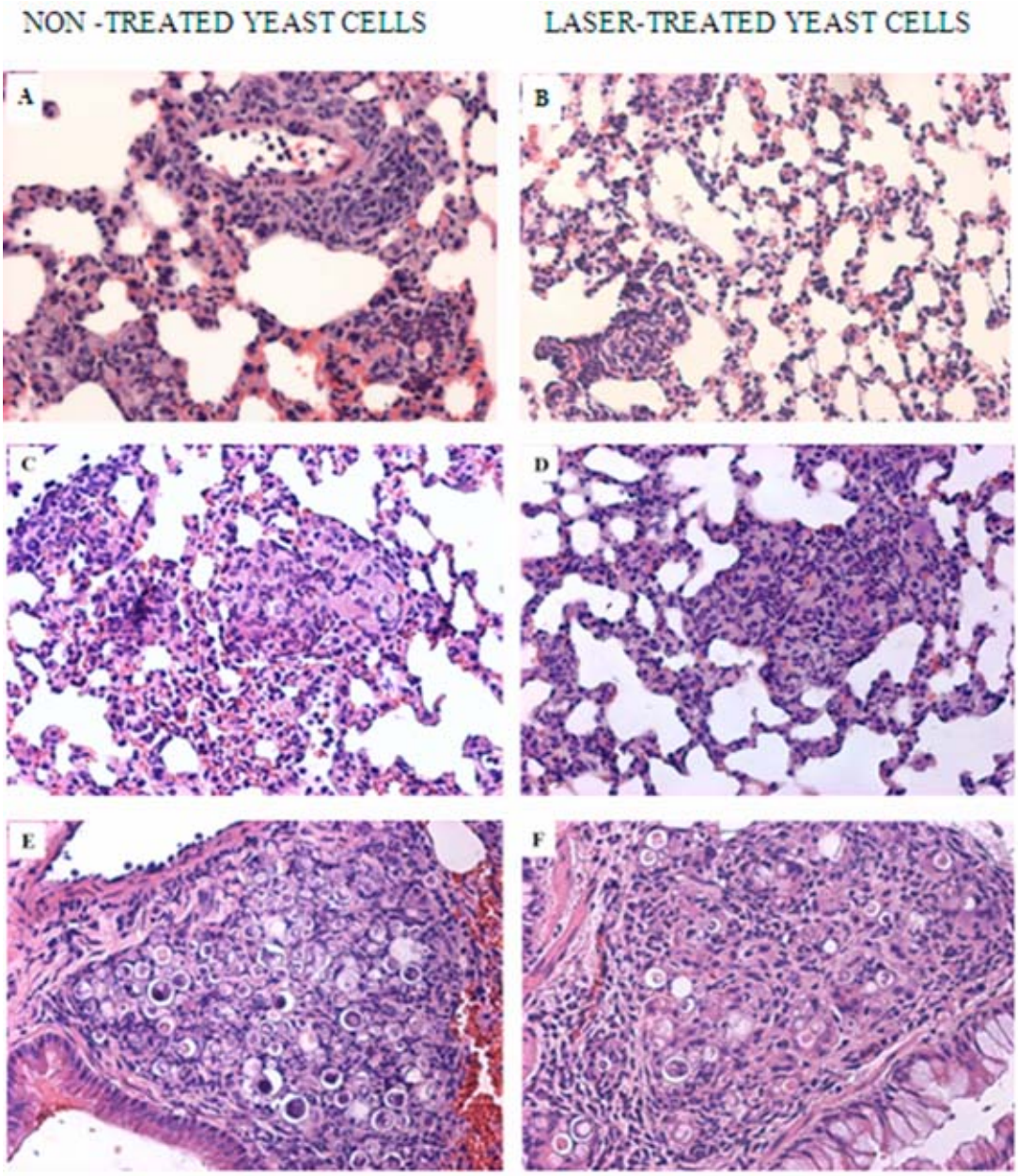

Figure 6. Representative histological findings in C57Bl/10 mouse lungs at 7 (A and B), 14 (C and D) and 30 (E and F) days post infection with laser-treated (right panel) and non-treated (left panel) *P. brasiliensis* yeast cells. At 7 days post-infection, a significant inflammatory infiltrate and the massive presence of macrophages in mice inoculated with non-treated yeast cells can be observed. At 14 and 30 days, however, histological changes are not different in the character or frequency of the pulmonary lesions in the animals that were inoculated with non- or laser-treated yeast cells. HE. Magnification: x 400.

P. brasiliensis yeast cells. These results partially disagree with those found by Andrade *et al.* [26] showing that *Pb* yeast cells lose their reproductive ability but retain the viability up to 21 days after gamma irradiation treatment.

Several cytological changes in HeNe laser-treated yeast cells of *Paracocididioi desbrasiliensis* were also observed in this study. However, we could not detect any DNA damage caused by HeNe laser treatment as reported earlier by Andrade and

his group [26] that has demonstrated extensive DNA fragmentation, along with nuclear alterations, while studying the same yeast cells treated with gamma irradiation. It does seem that the HeNe laser, contrary to gamma irradiation, has no direct effect on the nucleus of fungal cells but more studies are necessary to evaluate this matter.

A notable observation in the treatment of yeast cells with HeNe laser was the increased expression of gp43, the most important antigen of *P. brasiliensis*.

One hypothesis to explain this fact is that HeNe laser alters the mechanism that regulates the expression of the gp43 molecule. Alternatively, it could represent a strategy adopted by the fungus to cope with the detrimental process caused by HeNe laser treatment.

This increased expression of gp43 certainly must explain the *in vivo* pathogenicity of *P. brasiliensis* even after laser treatment. Both hypotheses, as well as their implications for the pathogenicity of the fungus, are presently being contemplated in our laboratory.

Previously, we have reported that HeNe laser applied to the paracoccidioidomycotic lesion increases the production of proinflammatory cytokines and the synthesis of extracellular matrix components, improving wound healing [8, 9, 10]. The data reported here showing that HeNe laser has also a direct action on the fungus corroborates our previous findings, providing further evidence that HeNe laser can be an adjunctive therapy during PCM treatment.

CONCLUSION

Although the laser treatment cannot be used as a vaccine in preventing paracoccidioidomycotic infection, it can be useful in clinical practice as an additive and synergistic treatment in combination with drugs in order to accelerate the healing of cutaneous lesions caused by *Paracoccidioides brasiliensis*.

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