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Research Article

Low-Level Laser Therapy: A New Complementary Therapeutic That Acts Against the Paracoccidioides Fungus and Improves Immune Performance

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Abstract

Paracoccidioidomycosis (PCM) is a disease with complex, difficult to treat clinical manifestations requiring long-term treatment with antifungals, which often leads to patients giving up and abandoning treatment, this makes fungal strains more resistant and hinders treatment. The synthesis of new compounds with effective antifungal action that shorten treatment time faces several challenges, therefore, Low-Level Laser Therapy (LLLT) has been shown to elicit a more effective immune response, proving to be a good therapeutic option for reducing the treatment time of PCM. Immunity in PCM is marked by intense recruitment of phagocytic cells, mainly Polymorphonuclear Neutrophils (PMN), which play a key role in activating an effective immune response against the fungus and the LLLT promotes more active cellular recruitment against the fungus.

Objectives: Here we evaluated the effect of LLLT on PMN obtained from subcutaneous infection, co-cultured with *Paracoccidioides Brasiliensis* (Pb).

Methods: Assaying cellular activity through the release of oxygen metabolites (ROS, H_2O_2, catalase), production of IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 and GM-CSF cytokines and also fungicidal activity.

Results: The results showed an increase in PMN activity and in the production of ROS, catalase and H_2O_2 when LLLT was applied to PMN co-cultures with Pb. Production of IL-4, IL-6 and IL-10 increased and the numbers of fungal colonies decreased when irradiated with LLLT. We also observed increase in the phagocytic activity of PMN co-cultured with Pb.

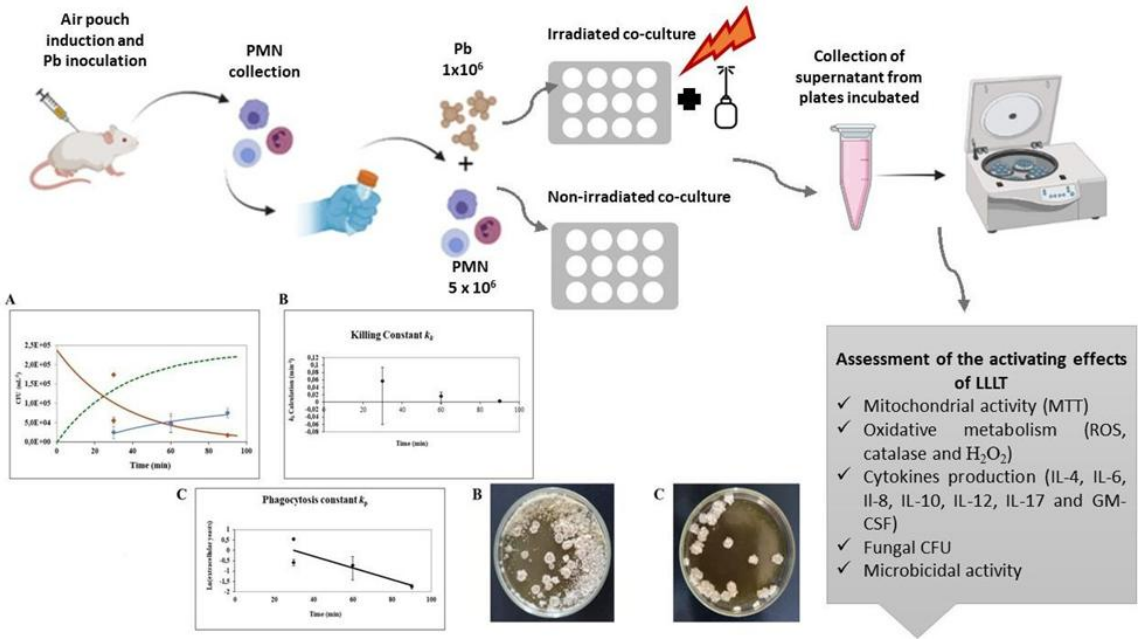
Conclusion: Therefore, the present study evaluated the effect of LLLT directly on PMN by directly irradiating these cells co-cultivated with Pb and showed that LLLT increases the metabolic activity of this cell population, resulting in increased competence in killing the fungus Pb and may act as a new complementary therapy against PCM.

Keywords: Low-Level Laser Therapy; P Brasiliensis; Neutrophils Activation; Increased Oxidative Metabolism Products; New Complementary Therapy

Significance Statement

- LASER activates oxidative burst through ROS, H_2O_2 and catalase production
- LASER induces secretion of regulator cytokines in vitro
- LASER increases phagocytic pathway in neutrophils
- LASER induces immune response to kill the fungus
- We propose Low Level LASER therapy to reduce fungal load

Graphical Abstract



Introduction

Paracoccidioidomycosis (PCM) is characterized as a granulomatous disease caused by thermodimorphic fungi, belonging to the genus *Paracoccidioides spp*. Most infections caused by PCM in Brazil are caused by the fungus Paracoccidiodes brasiliensis (Pb) and in certain regions strains in Latin America by such as *Paracoccidiodes lutzii* and other general [1,2]. The disease manifests itself in adults with the formation of lesions mainly in the lungs, but can cause involvement of other organs including and the lymphatic system, as well as lesions in mucous membranes and skin, mainly in regions of the face [3,4]. The primary immune response against this fungus plays a key role in the prognosis of the disease, in which the activation of Phagocytes, Mainly Neutrophils (PMN), has a central action in the development of an immune response capable of containing fungal dissemination and preventing the development of the chronic form of the disease [5,6]. PMN perform potent antimicrobial activity, through phagocytosis, degranulation and production of extracellular traps (NETs) [7]. PMN can activate protective immune responses against fungi including Pb, playing a role in the direct elimination of the fungus and also through interaction with other cell types, modulating the acquired immune response [5]. Previous studies show that the immune response against PCM tends towards a response profile in which a primary response activates a Th1 signalling pathway, inducing phagocytes to migrate to the site of infection and subsequently an activation of the Th2 pathway, enabling a response pathway that can worsen the disease, in which PCM can develop chronically [6]. The role of low-level LASER therapy (LLLT) has been described by our group in Pb infection as an additional therapy in the treatment of experimental PCM considering its potential fungicidal and cytokine activating effect and its role in modulating more efficient healing at the site of the injury [8]. Since the use of LLLT during Pb infection in an animal model can improve the performance of immune response cells, by activating oxygen metabolism phagocytes and the production and release of activating inflammatory cytokines. LASER therapy can improve the healing process of lesions caused by the fungus Pb [8-12]. Despite these earlier it is still unknown how LLLT can specifically modulate specific populations of immune response cells. Considering the important role of inflammation in the immune response against fungi, especially Pb, the present study aims to evaluate the use of LLLT directly on neutrophils by directly irradiating these cells maintained in culture.

Methodology

Animals

Six-week-old female Swiss mice weighing approximately 25 g obtained from the Federal University of Alfenas animal facility were used. All experiments were approved and conducted in accordance with the National Guidelines of the Animal Care Committee at the Federal University of Alfenas (CEUA Protocol: 07.1.365.53.8 /16/2018) and executed in triplicates. Additional information is filled in the ARRIVE guidelines 2.0 author checklist (Fig. 1).

Paracoccidioides Brasiliensis cultivation and preparation of fungal cell suspensions for infection

Yeast forms of fungi the highly virulent *Paracoccidioides Brasiliensis* (Pb18) strain [19] were grown in semi-solid Fava-Netto medium [13] and subcultured every 7 days. The fungal cells were washed with 0.9% sterile saline and centrifuged (Eppendorf 5804R) at 1300 RPM at 5°C, three times for 10 minutes. Fungal cell viability was determined by staining with the Janus Green B and the cell concentration was adjusted to 100% viable cells [6]. For inoculation, the yeast suspension was adjusted to 5×10^6 viable cells/mL.

Air-pouch model and mice infection

An air pouch was induced in the dorsal region of each mouse by subcutaneous injection of 2 mL of sterile air [6]. After air pouch formation, mice were inoculated with 0.1 mL of Pb18 suspension from 5×10^6 viable cells/mL and animals were maintained in animal facility for 10 days. Additional information about the model and cell collection can be found in the supplemental material.

Collection of PMN-rich cell suspension

Ten days post-infection, mice were euthanized with a lethal dose of anesthetics (0.5 mL of 10% ketamine chloride plus 2% xylazine solution). Following this, a skin incision was made and with the aid of a sterile glass pipette, cells were collected, transferred to a sterile glass and homogenized with RPMI medium supplemented with 10% Fetal Calf Serum (FBS) and transferred to tubes containing at 4°C. In this way, a suspension of cells rich in PMN was obtained [6].

PMN cell culture in co-culture with Pb

The cell suspension was quantified by counting in a hemocytometer and viability was determined using 0.2% Trypan Blue dye (Sigma). The PMN-rich cell suspension was adjusted to 5×10^6 cells/mL. Cells were cultured in 12-well plates and placed in contact with Pb for co-culture at a concentration of 1×10^6 cells/mL per well and incubated in a 5% CO_2 atmosphere at 37°C for 2, 6 and 18 hours. LASER irradiation was performed for 30 seconds before incubating the plates for 6, 12 or 18 hours. After the incubation period, the cells were centrifuged at 1780×g for 10 minutes and the supernatant and pellet were separated. Supernatants were used for determination of cytokines, proteins and oxygen metabolism [8].

LASER Irradiation

Twelve-well plates containing the cell suspension rich in PMN with Pb in co-culture were irradiated for 30 seconds before incubation for 2, 6 or 18 hours. LASER irradiation was carried out on the plate at a 90º angle with the bottom of the plate containing the cells. All irradiations were carried out in a dark environment to avoid interference from conventional light in the absorption of the LASER light wavelength by the cells. LLLT irradiation was performed using a Twin Flex LASER device (MMO, São Carlos, SP, Brazil) with a spot size of 0.04 cm^2. The LASER parameters were continuous near-infrared light (780 ηm) delivering 12.5 J/cm^2 with a total power of 50 mW; the total energy was 0.5 J per point (30 seconds) [14]. The LLLT parameters used in the study were previously described by our group [12].

Mitochondrial Activity Assessment

PMN-rich suspensions (5×10^6 cells/mL) with Pb in co-culture were pipetted into a 96-well plate (Corning) and 20 μL of 20% MTT (Sigma) was added. Thereafter, supernatants were removed, Dimethylsulphoxide (DMSO) was added and readings were taken at 540 ηm [15].

Effect of LLLT in PMN culture on the activation of oxidative metabolism products

Reactive Oxygen Species (ROS) quantification

The quantification of ROS was carried out by chemiluminescence assays. ROS capture was performed by determining the integrated light emission over seconds. In the luminol assay, cells were adjusted to 5×10^6 PMN cells/mL. Readings were performed using a luminometer (Promega-Glomax 20/20 Luminometer) and the chemiluminescent intensity was measured for 30 minutes [16].

Quantification of hydrogen peroxide (H_2O_2) levels

H_2O_2 release was measured using the phenol red oxidation method. The experiment was carried out according to the method described by Pick and Keisari [17]. Absorbance was evaluated using an ELISA plate reader (ANTHOS ZENITH® 200 rt) at 610

ηm. The absorbance conversion was carried out in μM H_2O_2 through deduction of a standard curve obtained with known concentrations of H_2O_2 (5-40 μM).

Quantification of Catalase Enzyme (CAT) levels

The activity of the Catalase Enzyme (CAT) was determined by measuring the decomposition of H_2O_2 per minute at a wavelength of 240 ηm. Results were expressed in U/mg of protein, with U corresponding to the enzymatic activity capable of promoting the hydrolysis of 1 μmol of H_2O_2 per minute [18].

Cytokines quantification

Cytokine concentrations were determined in cell supernatants at 2, 6 and 18 hours using commercially available ELISA kits for GM-CSF, IL-4, IL-6, IL-8, IL-10, IL-12 and IL-17, performed according to the manufacturer's instructions (PEPROTECH/ELISA Development Kit).

Viable Pb quantification through colony-forming units counting

The pellets from the subcutaneous air pouches were resuspended in 100 μL PBS and spread on petri dishes containing BHI culture medium supplemented with fetal bovine serum. Fungal growth was allowed to take place over a period of 12 days, during which colonies were counted daily [8].

Evaluation of microbicidal capacity of PMN against Pb

To evaluate PMN elimination capacity, the methodology proposed by Green, et al., and Parker, et al., adapted by Cerdeira, et al., with some modifications, was used [9,19,20]. This technique evaluates the ability of PMN to phagocytose and destroy microorganisms (Pb). Fungal suspensions were incubated at a concentration of 4×10^6 cells/mL with Hank's Balanced Salt Solution (HBSS) together with PMN at a concentration of 10^6 cells/mL in a CO_2 incubator at 37°C for 10, 30 or 90 minutes. After each incubation period, the respective group was treated with ice-cold PBS (pH 7.0) to cease neutrophilic activity, followed by differential centrifugation at 1780×g for 10 minutes. Pellets and supernatants were diluted in H_2O pH 11 to lyse PMN. After successive dilutions, samples were distributed in Petri dishes containing Brain Heart Infusion (BHI) medium supplemented with 10% Fetal Bovine Serum (FBS). Plates were incubated at 37°C and colony growth was quantified for 15 days. Results were expressed as graphs of phagocytosis constant (Kp) and killing constant (Kk), as both phagocytosis and killing by PMN follow first-order kinetics and can be calculated using a Lambert W function [19,20].

Statistical Analysis

Results were expressed as mean ± standard error of the mean. When comparing two group means, Student's t test was used, followed by Tukey post-test. GraphPad Prism 6 (GraphPad Software, Inc.) was used with a 5% significance level for all analyses. All experiments were performed in triplicate. For calculation of Kp and Kk, a Microsoft Excel® table available in the supplementary electronic material of Magon, et al., was used [21].

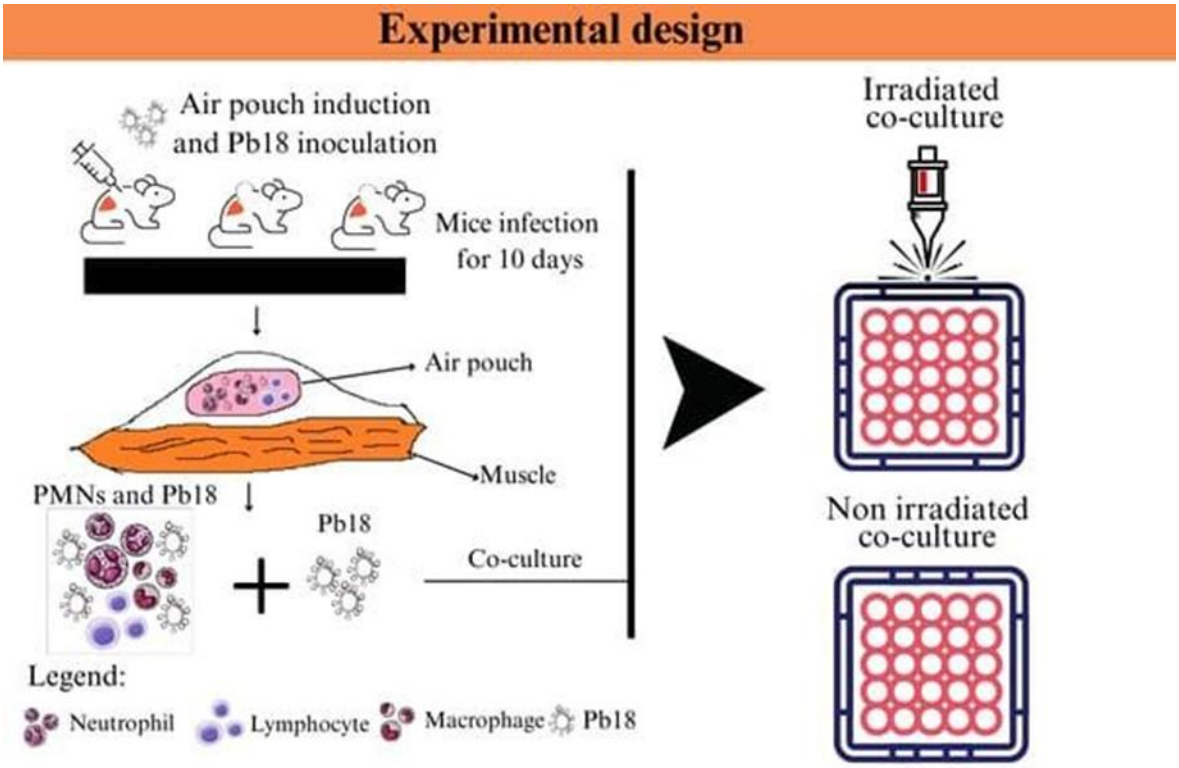


Figure 1: Experimental design of the *in-vitro* co-culture of PMN with *P. brasiliensis* and treatment with LLLT.

Results

Effect of LLLT on cellular activity and total protein production of PMN co-cultivated with Pb

Fig. 1 shows the results of the evaluation of mitochondrial activity (Fig. 2) and total protein production (Fig. 2) in non-irradiated co-culture (PMN with Pb co-culture) and in co-culture irradiated with LLLT (PMN with Pb co-culture + LLLT). In Fig. 2, there was a significant increase in cellular activity in the irradiated co-culture compared with the non-irradiated group ($p < 0.001$). Regarding total protein production (Fig. 2), a significant increase ($p < 0.001$) was observed at all evaluated times in the irradiated culture compared with the non-irradiated group.

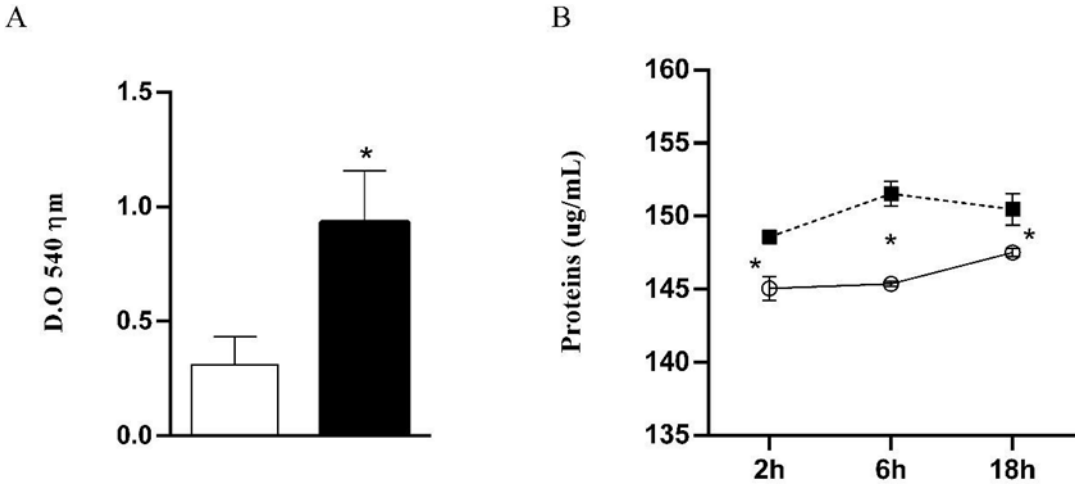


Figure 2: Effect of LLLT in PMN with Pb co-culture on mitochondrial activity (A) and total protein production (B). PMN with Pb co-culture (white bars and circles) and PMN with Pb co-culture irradiated with LLLT (black bars and squares). Results from all groups were expressed as mean ± standard deviation; all experiments were performed in triplicate. Groups were compared using Student's t test. Significance: $p < 0.001$ (*). The asterisk refers to values significantly different between co-culture and co-culture + LLLT.

Effect of LLLT on oxidative metabolism of PMN co-cultivated with Pb

Fig.3 shows the effect of LLLT on PMN co-cultivated with Pb on the release of oxidative metabolism products (Fig. 3).

In Fig. 3, which evaluated H_2O_2 production, a significant increase ($p < 0.001$) was observed in the irradiated co-culture compared with the non-irradiated group. Catalase production also increased significantly in the irradiated co-culture compared with the non-irradiated group (Fig. 3) ($p < 0.001$). A significant increase in ROS release was observed in the irradiated co-culture (Fig. 3) ($p < 0.001$).

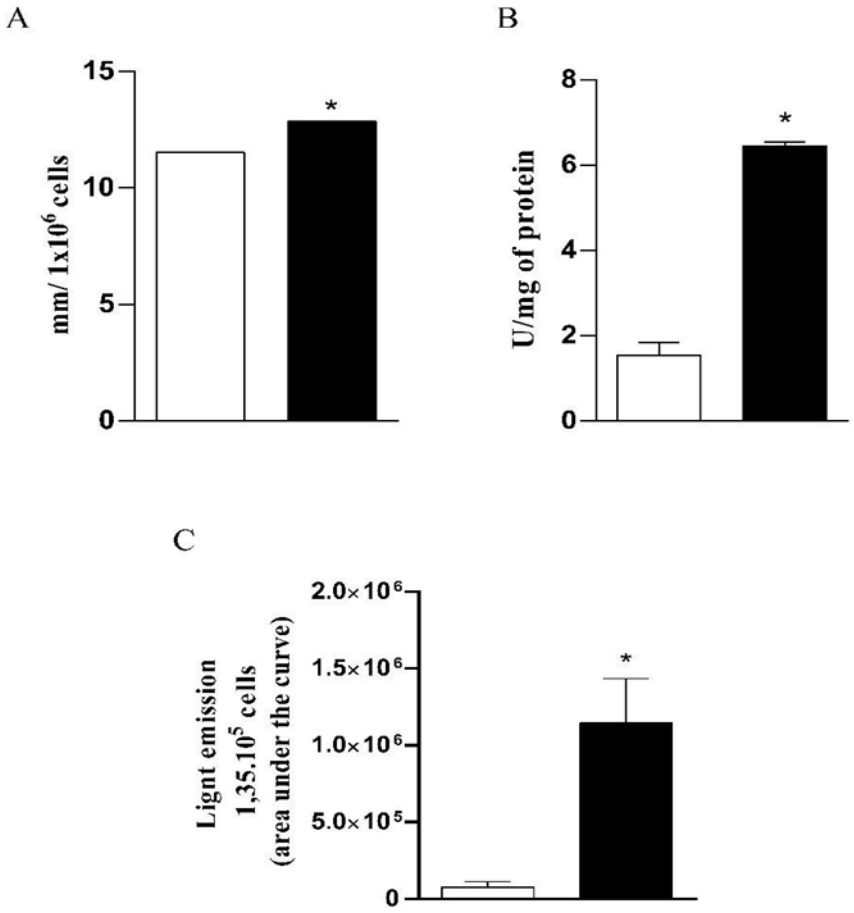


Figure 3: Effect of LLLT on PMN co-cultivated with Pb on oxidative metabolism products: H_2O_2 (A), catalase (B) and ROS (C). PMN with Pb co-culture (white bars) and PMN with Pb co-culture irradiated with LLLT (black bars). Results are expressed as mean ± standard deviation; all experiments were performed in triplicate. Groups were compared using Student's t test. Significance: $p < 0.001$ (*). The asterisk refers to values significantly different between co-culture and co-culture + LLLT.

Effect of LLLT on cytokine production of PMN co-cultivated with Pb

Fig. 4 shows cytokine release of IL-4 (A), IL-6 (B), IL-8 (C), IL-10 (D), IL-12 (E), IL-17 (F) and GM-CSF (G) in PMN with Pb co-cultures that were irradiated or non-irradiated with LLLT.

Cytokine production was measured at three time points (2, 6 and 18 hours). In Fig. 4, a significant increase ($p < 0.001$) in IL-4 release was observed at 2 hours in the irradiated culture compared with the non-irradiated culture. IL-6 release (Fig. 4) was significantly increased ($p < 0.001$) in the irradiated culture at 2 and 6 hours. IL-10 release (Fig. 4) showed a significant increase ($p < 0.001$) in the irradiated culture at 18 hours. IL-17 production was significantly decreased following LLLT exposure compared with non-irradiated cells at 6 hours ($p < 0.001$). IL-8, IL-12 and GM-CSF (Fig. 4 respectively) did not show significant differences between irradiated and non-irradiated co-cultures ($p > 0.05$).

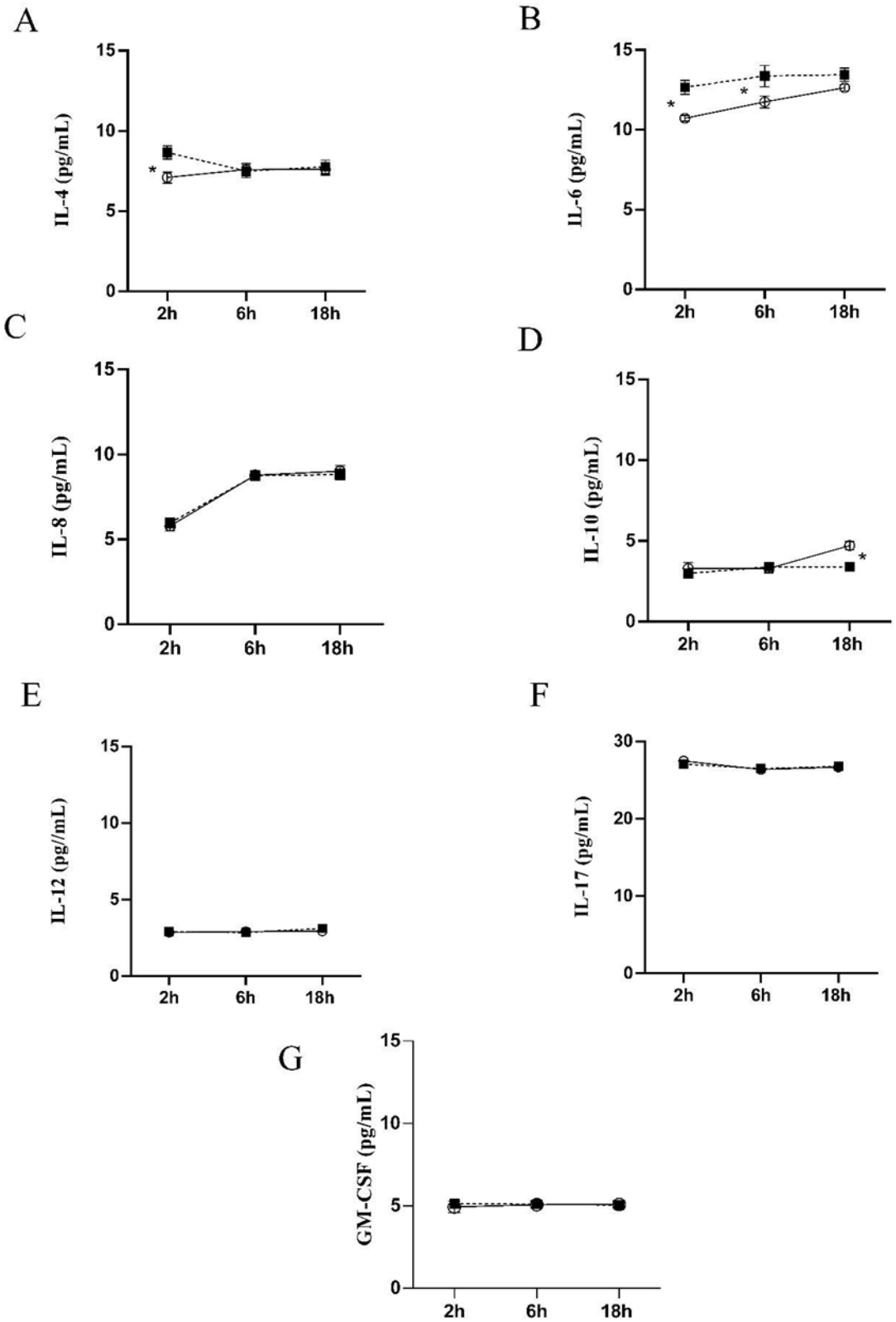


Figure 4: Effect of LLLT on PMN co-cultivated with Pb on cytokine production: IL-4 (A), IL-6 (B), IL-8 (C), IL-10 (D), IL-12 (E), IL-17 (F) and GM-CSF (G). PMN with Pb co-culture (white circles and continuous lines) and PMN with Pb co-culture irradiated with LLLT (black circles and dotted lines). Results are expressed as mean ± standard deviation; all experiments were performed in triplicate. Groups were compared using Student's t test. Significance: $p < 0.001$ (*). The asterisk refers to values significantly different between co-culture and co-culture + LLLT.

Effect of LLLT on the number of viable fungi in PMN co-cultivated with Pb

Fig. 5 shows fungal growth in PMN co-cultivated with Pb, either irradiated with LLLT or non-irradiated. Fig. 5 demonstrates a significant decrease in the number of Colony-Forming Units (CFU) in the irradiated co-culture compared with the non-irradiated co-culture ($p < 0.001$). Fig. 5 illustrate fungal growth in Petri dishes from non-irradiated co-cultures and LLLT-irradiated co-cultures, respectively.

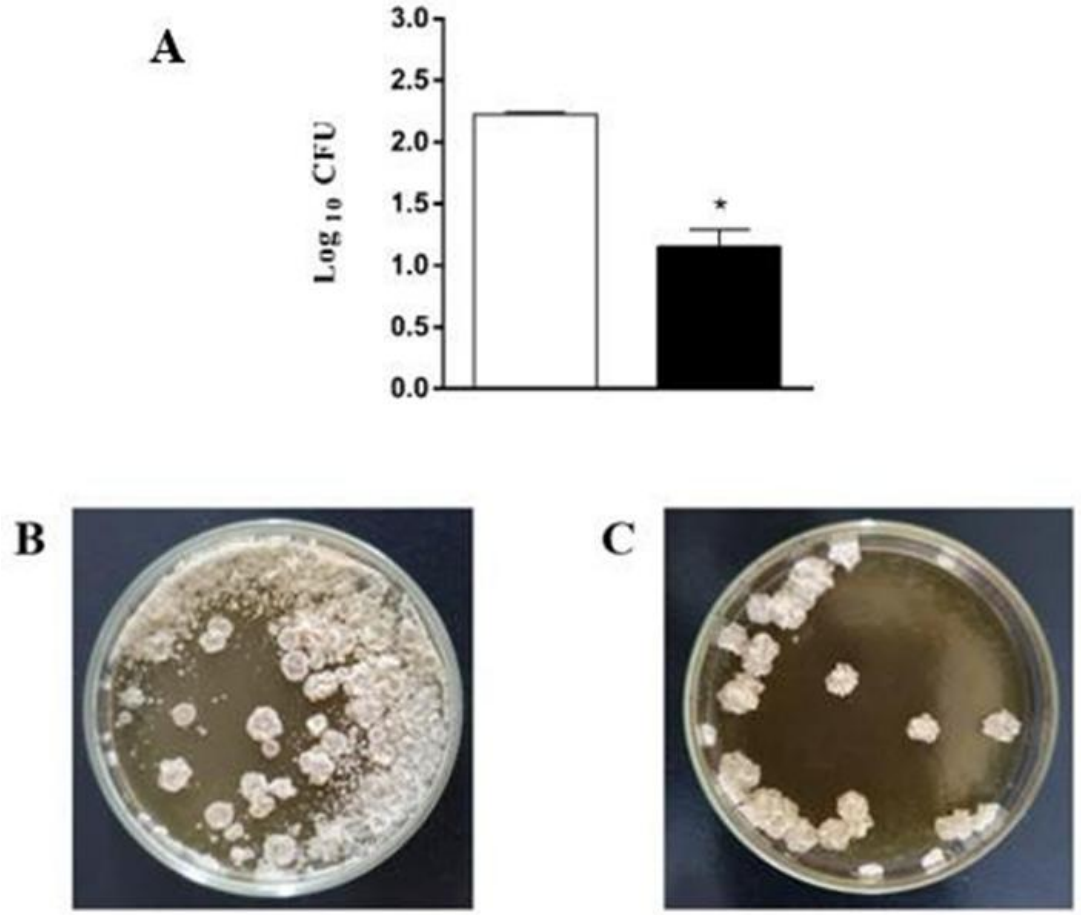


Figure 5: Effect of LLLT on PMN co-cultivated with Pb on the number of colony-forming units after 15 days of growth. CFU (A); PMN with Pb co-culture (white bars) and PMN with Pb co-culture irradiated with LLLT (black bars). Fungal growth from PMN with Pb co-culture (B) and from PMN with Pb co-culture irradiated with LLLT (C). Results are expressed as mean ± standard deviation; all experiments were performed in triplicate. Groups were compared using Student's t test. Significance: $p < 0.001$ (*). The asterisk refers to values significantly different between co-culture and co-culture + LLLT.

Evaluation of the phagocytic capacity and opsonization of PMN on Pb after LASER therapy treatment

Fig. 6 demonstrates the microbicidal capacity of PMN during co-cultivation experiments (PMN with Pb). The curves in Fig. 6 represent CFU numbers generated by calculation of the killing constant (Kk) (Fig. 6), which measures the ability of PMN to promote fungal death and the phagocytosis constant (Kp) (Fig. 6), which measures PMN phagocytic activity at 30, 60 and 90 minutes. The red curve represents extracellular fungi, the blue curve represents intracellular fungi and the dashed green line corresponds to the control group consisting of PMN from subcutaneous infection.

The results in Fig. 6 demonstrate that, over time, the number of extracellular CFU decreases, while the number of intracellular CFU increases after 60 minutes, indicating progressive phagocytosis of fungi by PMN. Additionally, a decrease in both the killing constant (Kk) and phagocytosis constant (Kp) was observed over time, suggesting a reduction in extracellular Pb and a corresponding increase in intracellular Pb (Fig. 6).

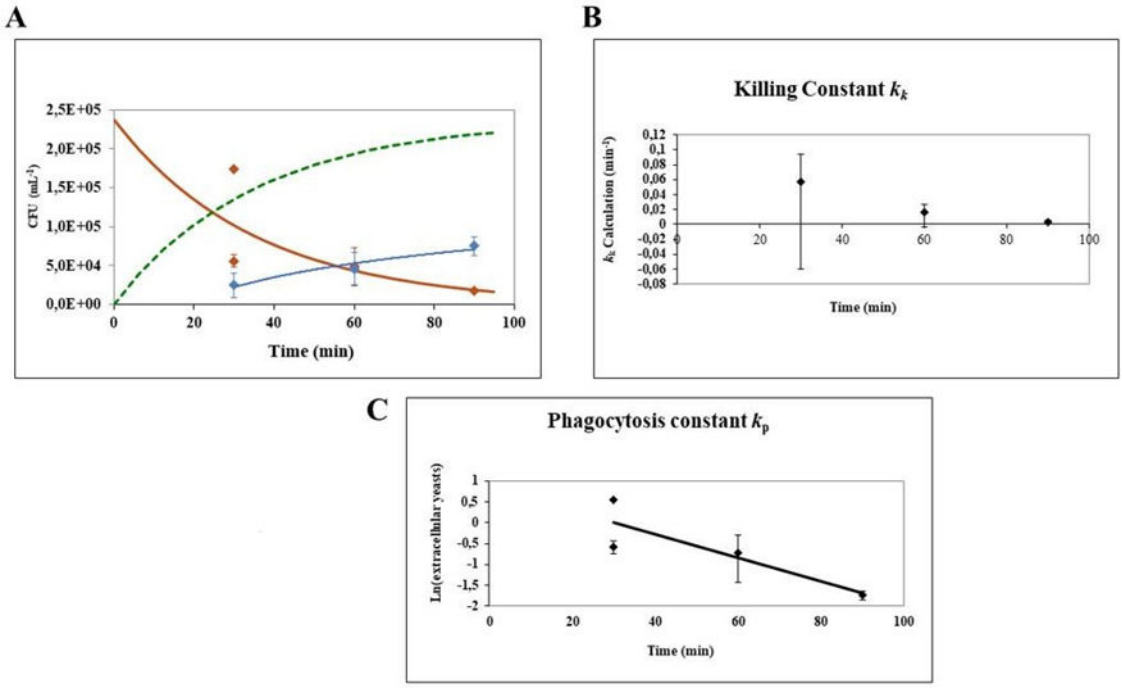


Figure 6: Evaluation of the phagocytic capacity of PMN on Pb following LASER therapy treatment. (A) Summary of PMN phagocytosis and killing capacity results; (B) killing constant (Kk); (C) phagocytosis constant (Kp). The red curve represents extracellular fungi, the blue curve represents intracellular fungi and the green curve represents PMN cells (control).

Discussion

Paracoccidioidomycosis (PCM) is a chronic fungal disease of great clinical importance in Latin America, caused by fungi of the genus *Paracoccidioides* [22]. The disease can progress to severe forms with pulmonary manifestations [4], lesions in peritoneal organs [2], involvement of the lymphatic system and wounds in the orofacial region [3]. Treatment in such cases is usually prolonged, requiring antifungal therapy for several months or even years in cases of disease recurrence [23].

Several medications and therapeutic approaches for the treatment of PCM have been recently described in the literature, aiming to demonstrate how their use can promote a more effective immune response against the fungus [24]. These new therapeutic approaches include the possibility of combining different medications [25] or even natural products [16], which may counteract some clinical aspects of fungal lesions and improve the patients' clinical condition [25].

Low-level laser therapy (LLLT) is one of the therapeutic alternatives that has shown promise in the treatment of lesions by accelerating the cicatrization process [26]. Studies in the literature have demonstrated the action of LASER on integumentary lesions in nails caused by fungi of the genus *Candida spp.* [27]. Another study demonstrated the use of LLLT in the treatment of lesions caused by sporotrichosis in wounds of domestic animals [28]. In addition, studies have shown activation of the immune response capable of inducing death of *P. brasiliensis* (Pb) in animal models [8], as well as the ability of LLLT to generate an immune response that promotes tissue remodeling, inducing increased healing at sites infected by Pb [12].

In the present study, LLLT was analyzed using a primary cell culture obtained through subcutaneous inoculation of Pb, which generates a large influx of inflammatory cells, predominantly Polymorphonuclear Neutrophils (PMN). These PMN were cultivated *in-vitro* and placed in contact with Pb and the resulting co-culture was irradiated with LLLT. This approach allowed evaluation of the ability of LLLT to activate PMN and promote lysis of fungal cells present in the culture. Our results showed that LLLT promoted an increase in cellular activity (Fig. 2) and induced PMN to secrete higher levels of total proteins (Fig. 2B). These proteins may be involved in PMN degranulation, resulting in the release of proteins into the extracellular environment that play a role in microorganism opsonization. In Pb infection, PMN are known to play an important role in developing a protective immune response, activating cytokines that promote the migration of phagocytes to the site of infection in both patients and experimental models [6]. Thus, increased protein production may reflect enhanced production of proteins involved in opsonization or increased cytokine secretion [6].

This activity becomes clearer when analyzing the data shown in Fig.3, which evaluated the effect of LLLT on the release of oxidative metabolism products. Increased release of Reactive Oxygen Species (ROS), Hydrogen Peroxide (H_2O_2) and catalase was observed following LASER irradiation of PMN cultures, indicating activation of the oxidative burst at the PMN level. These findings are consistent with reports by Kabela, et al., and Belambri, et al., who described that oxidative mechanisms begin with oxygen production by the NADPH oxidase enzyme complex during the respiratory burst [29,30]. Activation of this pathway involves changes in the localization and activity of NADPH oxidase components, resulting in H_2O_2 release, which is subsequently processed by catalase to generate oxygen ions [29]. Although phagocytes possess additional microbicidal mechanisms, such as antimicrobial peptides and enzymes, ROS generation during phagocytosis is considered a key mechanism in the destruction of invading pathogens [30].

Previous studies have reported that, in experimental models of PCM, LLLT increases the release of oxidative metabolism products, activating respiratory cascades and improving immune responses [6]. In *in-vivo* PCM models, LLLT has been shown to enhance the fungicidal capacity of immune cells such as PMN, improve healing of Pb-induced lesions [12] and activate cytokines leading to increased PMN recruitment and ROS release [6]. These observations are consistent with the findings of the present *in-vitro* study using PMN co-cultured with Pb and treated with LLLT, in which activation of the ROS metabolic pathway was evident.

In this study, LLLT did not significantly alter the production of cytokines such as IL-8, IL-12 and GM-CSF, which are known neutrophil activators [31]. However, increased production of IL-4, IL-10 and IL-6 was observed within the first hours of incubation and IL-17 levels increased after 18 hours in irradiated PMN-Pb co-cultures (Fig. 4). The increase in IL-17 is consistent with findings reported by Mamoni, et al., in patient studies [32]. These cytokine profiles differ from those previously observed in *in-vivo* animal models [12], in which increased production of all inflammatory cytokines was described. In contrast, the present study demonstrated increased production of anti-inflammatory cytokines IL-4 and IL-10. This difference may be attributed to the limitations of the *in-vitro* culture system, which does not allow recruitment of other phagocytes to the infection site, thereby restricting the release of PMN-activating cytokines. Conversely, increased levels of regulatory cytokines such as IL-6 were observed. The late increase in IL-17 may be related to enhanced phagocytic activity and opsonization aimed at fungal destruction. These findings are consistent with other studies demonstrating that LASER therapy tends to induce anti-inflammatory and regulatory immune responses, including increased cytokine production associated with Treg-mediated pathways [33,34]. Similar regulatory cytokine activation has been reported in PCM cytokine profile studies [35].

The increased release of oxygen metabolites and the presence of IL-17 at later incubation stages may be intrinsically related, contributing to the observed reduction in Colony-Forming Units (CFU) (Fig. 5) and consequently, to a decrease in the number of viable fungi in LLLT-irradiated co-cultures, as also reported by Meloni-Bruneri, et al. [6]. The microbicidal activity of LLLT was further evaluated in this study and Fig. 6 demonstrates the phagocytic capacity of PMN in destroying Pb. As incubation time increased following irradiation, intracellular fungal counts increased while extracellular fungal counts decreased, indicating enhanced phagocytosis, reflected by changes in the phagocytosis constant (Kp), as described by Hampton, et al. [36]. Over time, both Kp and the killing constant (Kk) decreased, which is expected given that most Pb had already been phagocytosed, as described in the analytical model proposed by Green, et al. [19]. These findings demonstrate enhanced fungicidal capacity of PMN following LLLT, supported by increased activity of oxygen metabolites such as ROS, catalase and H_2O_2, resulting in increased PMN activity and fungal lysis, as confirmed by CFU analysis.

Previous data from our group demonstrated that LLLT induces oxidative burst in neutrophils and promotes cytokine release capable of regulating inflammatory mechanisms in PCM. In the present *in-vitro* model, PMN obtained from subcutaneous Pb infection and subsequently co-cultured with Pb and exposed to LASER irradiation exhibited enhanced phagocytic activity and increased microbicidal responses mediated by oxygen metabolites, resulting in fungal death and reduced fungal load.

Conclusion

Using an *in-vitro* model, we demonstrated that LASER irradiation activates oxidative burst through increased production of ROS, H_2O_2 and catalase, promotes secretion of regulatory cytokines, enhances neutrophil phagocytic activity and induces killing of *P. brasiliensis*, resulting in reduced fungal load. Therefore, low-level LASER therapy may be considered a complementary therapeutic approach in the treatment of PCM.

Conflict of Interest

All authors declare that they have no conflict of interest.

Funding details

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Ethical Approval

This article contains studies involving animals and complies with the ethical standards of the Animal Ethics Committee (CEUA) of the Federal University of Alfenas, Brazil (Protocol number 0005/2020).

Author Contributions

All authors contributed equally to the study.

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