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Fluorescence spectroscopy of *Candida albicans* biofilms in bone cavities treated with photodynamic therapy using blue LED (450 nm) and curcumin

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ABSTRACT

Fluorescence spectroscopy may assisst in the diagnosis and control of infectious processes associated with bone lesions of the oral cavity. The aim of this study was to analyze, through fluorescence spectroscopy, *Candida albicans* biofilms formed in artificial bone cavities treated with photodynamic therapy (PDT) mediated with 450-nm blue light-emitting diode (LED) and curcumin. Another aim of this study was to analyze the existence of a correlation between the effectiveness of the photodynamic treatments and the fluorescence spectroscopy images. Artificial bone lesions ($n = 40$) were made in bovine bones and inoculated with standard suspensions of *Candida albicans* (ATCC 18804) for biofilm formation (14 days / 36 °C ± 1 °C). The 40 specimens were distributed among four experimental groups ($n = 10$): L-C- (control), L + C- (LED for 5 min), L-C+ (curcumin for 5 min), and L + C+ (PDT). Aliquots of 100 µL were collected from the bone cavities after treatments and were seeded in duplicate on Sabouraud dextrose agar for 24 h at 36 °C ± 1 °C and the colony-forming units (CFU/ mL) were counted. Before and after each treatment, the specimens were subjected to spectral fluorescence and the images were compared using the Image J program. The $\log_{10}$ CFU/mL were compared with Kruskal-Wallis and Dunn's Multiple Comparison post-test (significance level at 0.05). The fluorescence histogram values before and after treatment were compared using Wilcoxon test (95%).The correlation between *Candida albicans* $\log_{10}$ CFU/mL and the number of the fluorescence red pixels spectroscopy was verified using Spearman correlation test. The reduction of *Candida albicans* $\log_{10}$ CFU/mL in the L + C+ (PDT) group was the most relevant and the fluorescence spectroscopy was correlated to the microbiological result. It was concluded that there was a consistency between the number of *Candida albicans* $\log_{10}$ CFU/mL and the red pixel data of the fluorescence images, demonstrating that the fluorescence diagnostic device reflects the true microbiological condition of *Candida albicans* biofilms in the bone cavities during the pre-treatment and post-treatment, thus providing the clinician the ability to dynamically, simply, and instantaneously verify the performance of the treatment used.

1. Introduction

Optical methods and techniques have been developed aiming at both clinical treatment and diagnosis of illnesses and have brought numerous benefits to various medical specialties [1–3]. The concept of photodynamic diagnosis based on the observation of endogenous or exogenous fluorescence emitted by the molecular structures present in biological tissues when irradiated by ultraviolet light. The mechanism of images acquisition by optical fluorescence is based on the absorption of energy of a photon by an atom or molecule causing the re-emission of this photon with lower energy according to the characteristics of the atom or molecule responsible for the phenomenon [3–5]. Microorganisms contain substances such as porphyrins, which are observed in fluorescence spectroscopy and differ from the fluorescence

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characteristics of the biological tissues of humans and animals, thus becoming a relevant tool to conduct treatments and verify the effectiveness of the techniques used and propaedeutic employed [1].

Among the many possibilities of technologies use that employ optical fluorescence for diagnosis, biofilm control, and contamination, is an interesting application for the microbial control of infectious processes associated with bone lesions of the oral cavity [2,3,6,7].

In the oral cavity, the most appropriate therapeutic approach for infections associated with the presence of biofilm is its removal, which, in the cases of gingivitis and periodontitis, can be performed using conventional methods of oral hygiene or clinical intervention [6,7]. In endodontic infections, the biofilm penetrates the interior of the dentinal tubules, rendering it difficult to remove them by biomechanical preparation, and thus, it must be performed using chemical compounds with proven antibacterial activity, such as sodium hypochlorite (0.5% or 1%), chlorhexidine digluconate etc. [8–10]. In some cases, in inaccessible areas, clinical and surgical removal of the biofilm and/or antibiotic therapy is necessary, which, in addition to side effects and delayed results, may stimulate bacterial resistance when improperly used [8,11]. Currently, researches are attempting to determine adjuvant modalities of antimicrobial treatment with less possibility of side effects for the individual and with lower chances of resistant species generation, such as photodynamic therapy (PDT) [8,10–13].

In recent decades, the incidence of *Candida albicans* in endodontic infections has been prevalent, since it is related to primary and refractory infections, and there has been a renewed interest in PDT for potentially pathogenic fungi, especially *Candida albicans*, which is a common inhabitant of the mouth, throat, digestive tract, and skin [11–14]. Radcliffe et al. [15] reported that there is little information on the persistence and survival of microorganisms after endodontic treatment. According to these authors, *Candida albicans* is associated with the clinical cases of persistent endodontic infection and treatment failure [11–13,15]. *Candida* species have been assigned a significant persistence in periapical infections, which compromise the bone structure of the region, rendering treatment success even more difficult. However, the most significant aspect is that when the species is present, there is hardly an effective response to conventional treatment—a procedure that largely eliminates other microorganisms [12].

Recent studies in the literature demonstrate the potential use of PDT on *Candida* biofilms species using lights of different characteristics and diverse dyes both in dentistry and other medical areas [11–14,16]. Of note is the study conducted by Rosa et al. [14], that observed the potential of using blue light-emitting diode (LED) individually for the elimination of *Candida* biofilms in compact bones, demonstrating the interest of current research in investigating the effectiveness of technologies and photonic therapies for the biofilms elimination in different bone tissues. One of the photosensitizing dyes that has emerged in recent years due to its antimicrobial potential in PDT is curcumin, which is a compound isolated from *Curcuma longa*. Clinical studies have proved that curcumin is non-toxic, has a broad absorption peak in the 300–500-nm range, and has potential as a photosensitizer for PDT in the treatment of infections. Additional positive attributes include its low cost and simple handling [13].

Accordingly, the present study aims to analyze, using fluorescence spectroscopy, the *Candida albicans* biofilms formed in artificial bone cavities treated with PDT mediated with 450-nm blue LED and curcumin. Another aim of this study is to analyze the existence of a correlation between the effectiveness of the photodynamic treatments and the fluorescence spectroscopy images.

2. Material and methods

2.1. Preparation of specimens

Forty specimens were prepared from bone pieces of bovine ribs from butchers discard. The bone pieces were sectioned into specimens with length of 1.5 cm each using a diamond disc (Super Tech TM, China). Artificial cavities were made using a spherical diamond drill with the diameter of 1 cm. Each specimen was cleaned with hydrogen peroxide 10%, water proofed with chemically activated acrylic resin, and autoclaved at 121 °C for 15 min.

2.2. Contamination of specimens

The specimens were distributed into four groups (n = 10): L-C- (control), L + C- (LED), L-C+ (curcumin), and L + C+ (PDT) in a sterile petri dish. The artificial cavities were filled with 750 µL of Sabouraud dextrose broth (Difco, Detroit, USA) and contaminated with 100 µL of a standardized suspension of 1×10^6 cells/mL *Candida albicans* (ATCC 18804) in a spectrophotometer (Biospectro, SP - 220, Brazil) at 530 nm and optic density (OD) of 0.284. The medium was homogenized using a volumetric micropipette and incubated in a BOD (Solab Scientific, Piracicaba - SP, Brazil) for 14 days at 36 °C ± 1 °C for biofilm formation. The artificial cavities were filled daily with Sabouraud dextrose broth for the maintenance of nutritional conditions and for the formation of biofilms, according to the methodology established by Rosa et al. [14].

2.3. Experimental conditions

After biofilm formation, the cavities were treated according to the groups proposed in the study. In the blue LED group (L + C-), the specimens were subjected to light for 5 min using a prototype (Prototype, Projeto Finep/ Gnatus LED Edixeon, Edison Opto Corporation, New Taipei City, Taiwan) at 450 nm, 67 mW/cm^2, and 20.1 J/cm^2. In the group (L-C+) with specimens submitted exclusively to curcumin, the cavities were filled with a solution of curcumin (FS) (PDT Pharma, Cravinhos-SP, Brazil) of 1.5 g/L (2% ethyl alcohol), which stayed for 5 min in a protected environment of light exposure. In the PDT group, curcumin was used with the same parameters as those of the L-C + group and, after the removal of FS, irradiation with the parameters of the L + C- group were performed. After the treatments, the bone cavities were filled with sterile saline (0.9% NaCl) and washed ten times. After the detachment of biofilm, 100 µL was cultured in Sabouraud dextrose agar at 10^{-1}, 10^{-2}, and 10^{-3} dilutions and incubated in the BOD at 36 °C ± 1 °C for 24 h. The control group received no treatment.

2.4. Fluorescence spectroscopy

All the specimens were evaluated with an auxiliary diagnostic exam for the identification of microbial biofilms in the bone cavities using fluorescence spectroscopy at 405 nm (Evince - MMÓptics São Carlos - SP, Brazil), with a 400-nm ± 10-nm ultraviolet LED light source with a maximum luminous intensity of 40 mW/cm^2 ± 20%, optical filters (band pass 420-nm, reflection dichroic in the band 350-nm to 475-nm and transmission in the band 475-nm to 800-nm), a set of seven lenses for focusing the LEDs, and a reflector to obtain the illumination intensity. The digital images were analyzed using Image J software (Image Processing and Analysis in Java, 2009). The red pixels corresponding to the biofilms were analyzed using the execution function of an RGB histogram. The value used was the number of red pixels at the mid-point of their concentration in the histogram R (red).

2.5. Statistical analysis

Adherence to normality and homoscedasticity assumptions were verified using Shapiro-Wilk normality test. Kruskal-Wallis with Dunn's Multiple Comparison post-test (significance level at 0.05) was made to *Candida albicans* $\log_{10}$ CFU/mL comparisons. The fluorescence histogram values before and after treatment were compared using Wilcoxon test (95%). The correlation between *Candida albicans* $\log_{10}$ CFU/mL and

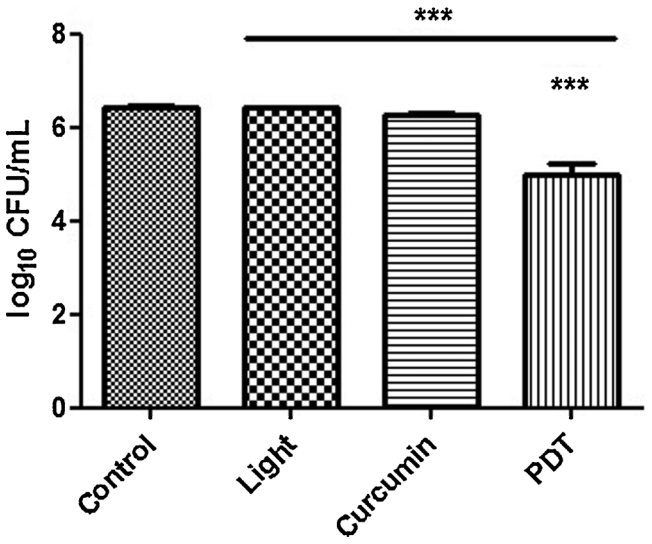

Fig. 1. Mean values, expressed in $\log_{10}$ CFU/mL, and the standard deviation, obtained using Kruskall-Wallis (5%) test.

the number of red pixels of the fluorescence spectroscopy was verified using Spearman correlation test. All tests were made with GraphPad Prism 5 for Windows.

3. Results

3.1. Effectiveness of Photodynamic Therapy in relation to other treatments

The comparison of the proposed treatments showed that the PDT (L + C+) group present significant statistical difference ($p < 0.05$) compared with control (L-C-), light alone (L + C-) and only curcumin (L-C+) groups, showing that PDT (L + C+) promoted more evident reduction in *Candida albicans* $\log_{10}$ CFU/mL (Fig. 1).

3.2. Fluorescence spectroscopy analysis

The value of the total number of red pixels demonstrated in the R histogram (red) was used to compare the images before and after the treatments. There was a significant difference ($p = 0.01$) only in PDT group (L + C+) (Fig. 2). In the LED group (L + C-), a reduction in the number of red pixels after the treatment was observed, which was not statistically significant ($p = 0.08$). In the curcumin group (L-C+), it was observed that, after treatment, there was an increase in the number of red pixels.

3.3. Correlation between Fluorescence and antimicrobial activity

It could be verified that there is a high positive correlation between the data obtained from fluorescence and *Candida albicans* $\log_{10}$ CFU/mL

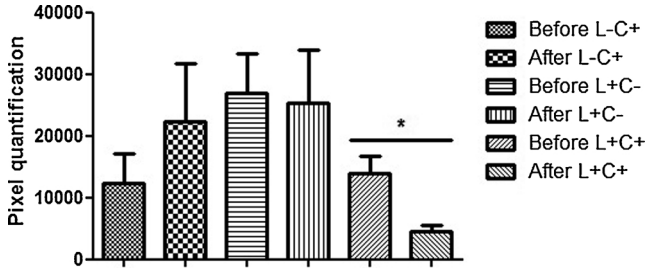

Fig. 2. Fluorescence values, expressed in red pixels of the image and standard deviation obtained with Wilcoxon (5%) test. L-C- = control, L + C- = LED for 5 min, L-C+ = curcumin for 5 min, and L + C+ = PDT. * p value.

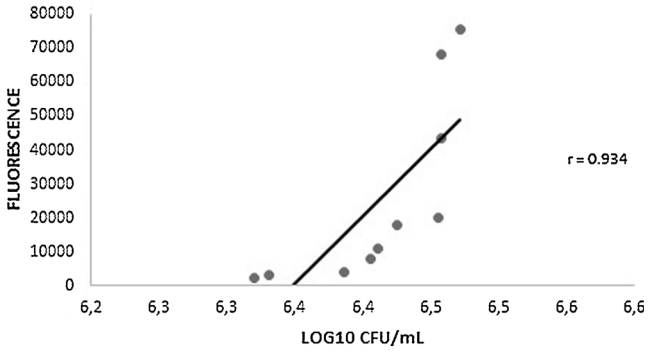

Fig. 3. Spearman correlation test between the means, expressed in units of $\log_{10}$ CFU/mL, and fluorescence, for the L + C- group.

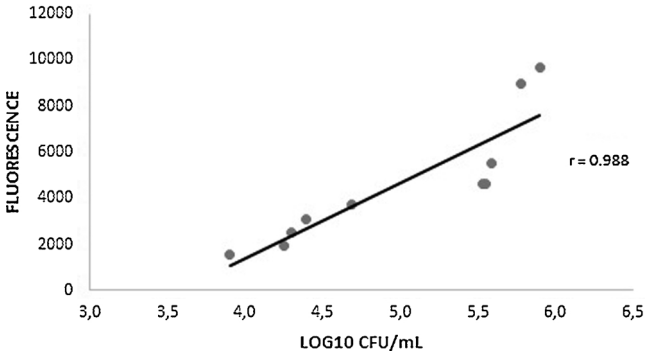

Fig. 4. Spearman correlation test between the means, expressed in units of $\log_{10}$ CFU/mL, and fluorescence, for the L + C + group.

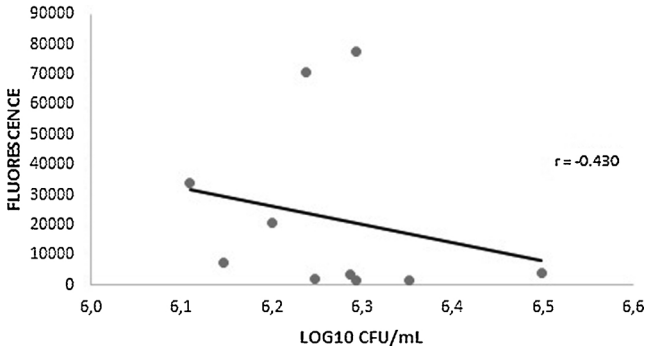

Fig. 5. Spearman correlation test between the means, expressed in units of $\log_{10}$ CFU/mL, and fluorescence, for the L-C + group.

in the L + C- ($r = 0.934$) and L + C+ ($r = 0.988$) groups, as shown in Figs. 3 and 4. However, in the data obtained with the L-C + group, a negative correlation ($r = -0.430$) was observed between the fluorescence values and $\log_{10}$ CFU/mL (Fig. 5).

4. Discussion

An optical fluorescence device with ultraviolet LED sources has already been used in dental clinics, since it is a relevant auxiliary diagnostic method owing to its precision, sensitivity, non-invasive nature, speed, safety and simplicity in use [2,3,6,7]. By attaching a camera to the device, either from a smartphone or from the device itself, it is possible to transfer the images to a computer where numerous possibilities of unfolding the images exist in order to enable the understanding of the fluorescence of different microbial tissues, lesions, and biofilms.

In dentistry, optical fluorescence devices are employed to accurately

diagnose incipient caries, exposed cementum junction, dental enamel demineralization, restorations, prostheses infiltration, and even small cracks [6,7], but little scientific evidence exists on the use of these devices for the study and diagnosis of biofilms and infectious lesions in bone structures of the maxillo-mandibular complex.

The literature also shows other applications of fluorescence optical diagnostic devices. Bachmann et al. [17] studied its applicability to thyroid surgery and observed that it is possible to differentiate malignant lesions from benign lesions using fluorescence as a diagnostic method. Manoel et al. [18] carried out a study where they proved the efficacy of fluorescence for diagnosis, photo-aesthetic treatment, and monitoring of alopecia. Pratavieira [19] analyzed the use of optical images for the early diagnosis of neoplastic skin lesions and showed optically different areas in malignant lesions using the fluorescence device.

However, numerous possibilities of use of these diagnostic devices should be explored in the field of health. Accordingly, the present study was developed for the analysis of spectral fluorescence diagnosis of *Candida spp*. biofilms, a species of yeast related to numerous types of lesions of the maxillo-mandibular complex. Among the lesions in bone tissues of dental interest, the species may be associated with bone lesions of endodontic etiology, perimplantites, periradicular lesions etc. These lesions, when associated with the *Candida* species, are difficult to treat and easily recidivate.

Another important feature of the device is it use for follow-up treatments. In the present study, *Candida albicans* biofilms were analyzed using spectral fluorescence before, during, and after the antimicrobial treatment by PDT with 450 nm blue LED associated with curcumin photosensitizer. There is no evidence in the literature that diagnostic fluorescence devices have been used in clinical or laboratory studies to analyze this type of biofilm in bone structures, and the results and analyses of the present study are therefore of significant importance to the field of health.

Although there are no studies with similar characteristics, Miyatake et al. [20] performed a study using fluorescence diagnosis to follow transoperative brain tumors and referred to the technique as "photodynamic diagnosis." In the afore mentioned study, the authors showed that some tumors showed red fluorescence, which is especially useful for the removal of the infiltrative portion in bone and normal parenchyma, and discussed the high quality of the technique for guided surgeries of malignant tumors.

Sieroń et al. [4] made important considerations about fluorescence photodynamic diagnosis. According to the authors, the photodynamic diagnosis is focused on five targets: detection for the prevention of precancerous changes of malignant transformation, detection of neoplastic tissue in the early stages for rapid removal, prevention of the expansion and the detection of cancer recurrence, monitoring of therapy, and the possibility to exclude neoplastic disease.

Regarding the results of PDT on *Candida albicans* biofilms in bone cavities, the present study observed that the PDT, with the parameters used, reduced 1.46 $\log_{10}$ CFU/mL of *Candida albicans*, in contrast to the case of using only 450 nm LED or curcumin for 5 min. The use of PDT with blue LED has demonstrated efficient results in other studies. Alves et al. [13], investigating the efficiency of the 460-nm LED associated with curcumin in the lethal photosensitization of *Candida albicans*, observed that the PDT with LED light and curcumin was efficient when compared to the experimental controls. The data showed that the association of curcumin and LED presented toxicity to the *Candida albicans* cells under the irradiation parameters used by the authors.

Rosa et al. [14] carried out a study observing the effectiveness of the 455 nm LED in the elimination of *Candida albicans* and *Staphylococcus aureus* biofilms on bone tissues. They concluded that, after application for 10 min, there was a significant reduction of the microorganisms biofilms. Despite the good results obtained by Rosa et al., in the present study, the blue LED did not significantly reduce the biofilms of *Candida albicans* from the bone cavities, perhaps owing to the characteristic of the spongy bone trabeculated that prevents the penetration of light into deep areas.

In order to dynamically monitor the situation of *Candida albicans* biofilms developed in the bone cavities, photographs from the fluorescence devices were obtained both for the control specimens and for the groups specimens treated with curcumin, blue LED only, and with PDT. In the fluorescence images of the control group, it was possible to observe the diffuse presence of red areas (indicative of contamination) in a large part of the bone cavity, in a manner consistent with the expressive number of *Candida albicans* $\log_{10}$ CFU/mL collected from these cavities in this group. Similarly, images consistent with the number of *Candida albicans* $\log_{10}$ CFU/mL were observed both in the group that the LED was used alone and in the PDT group, in the latter case, the least number of red dish areas were observed and in some specimens, red images were not observed. In the group where only curcumin was used, the red pixel value after the use of curcumin appears higher than the value of the previous period, but this condition is not accurate, since there was masking of the fluorescence owing to the brightness characteristic of the photosensitizer when visualized by this technique, which leaves the visual field whitish almost in its entirety.

As evident from the results of the study, there was a consistency between the number of *Candida albicans* $\log_{10}$CFU/ mL and the red pixel data of the fluorescence images, demonstrating that the fluorescence diagnostic device reflects the true microbiological condition of *Candida albicans* biofilms in the bone cavities during pre-treatment, treatment, and post-treatment, providing the clinician the ability to dynamically, simply, and instantaneously verify the performance of the treatment used.

Studies in the literature on the use of fluorescence for the diagnosis of biofilms and monitoring of treatments are scarce, which motivated the undertaking of the present study. However, studies that analyze other aspects of the fluorescence of these biofilms in bone infections are necessary and will contribute to the foundation of the use of fluorescence diagnosis in routine clinical dentistry and even in other health areas.

5. Conclusion

It was possible to conclude that:

(a) there is a high positive correlation between fluorescence spectroscopy and *Candida albicans* $\log_{10}$ CFU/mL both in the group where the LED was used alone and in the PDT group;
(b) It is not possible to consider fluorescence spectroscopy in the group where curcumin is used alone as a result of white fluorescence in the image, which over shadows the entire field of vision;
(c) PDT, under the parameters used, reduced 1.46 *Candida albicans* $\log_{10}$ CFU/mL, in contrast to 450 nm blue LED or curcumin for 5 min;
(d) there was consistency between the number of *Candida albicans* $\log_{10}$ CFU/mL and the red pixel data of the fluorescence images, demonstrating that the fluorescence diagnostic device reflects the true microbiological condition of *Candida albicans* biofilms in the bone cavities during the pre-treatment, treatment, and post-treatment, providing the clinician the ability to verify the performance of the treatment employed in a dynamic, simple, fast, and instantaneous way.

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