Original Research Article

In vitro effect of the association of therapeutic ultrasound to fluconazole on the growth inhibition of *Candida albicans*

ABSTRACT

Aims: The present work evaluated the synergistic effect of therapeutic ultrasound associated with fluconazole on *Candida albicans* in vitro.
Place and Duration of Study: The experiments were carried out at the Phytopathology Laboratory at the Federal University of Acre (UFAC) and the Microbiology Laboratory at the Estácio Meta University Center in Rio Branco City, State of Acre, Brazil.
Methodology: The experiments used a standard strain of *Candida albicans* (ATCC 10231). Planktonic suspensions of *C. albicans* were prepared and exposed to ultrasonic waves at frequencies of 1 MHz and 3 MHz, and intensity of 1.0 W/cm^2, with application times of 5, 10, and 15 minutes. The treatment groups were ultrasound (UST) alone, Fluconazole alone (FLU), ultrasound combined with Fluconazole (UST + FLU), and a control group (no UST, no FLU). After 24 hours, a count of the Colony Forming Units (CFUs) was performed.
Results: According to the results, a greater growth inhibition was observed in treatments (UST + FLU) at both frequencies for all three application times. It indicates that, at least in the conditions we used, therapeutic ultrasound combined with fluconazole inhibited the growth of *C. albicans* in vitro. The antimicrobial effect of this treatment was greater than the one with UST alone and with fluconazole alone.
Conclusion: The association of antifungals with ultrasound can be considered a promising strategy to treat infections caused by fungi, allowing a reduced therapeutic period.

Keywords: C. albicans, Ultrasound, Fluconazole, Fungi.

1. INTRODUCTION

Fungal infections caused by yeasts have been increasing in recent years [1] and due to its high morbidity and mortality rate, especially in immunocompromised patients, it has gained clinical importance [2]. Candidiasis is among the most recurrent yeast fungal infections. This disease is caused by the fungus of the genre *Candida* and mainly by the species *C. albicans* [3]. *C. albicans* is a diploid fungus and can be found as an inhabitant of the gastrointestinal and genitourinary tracts of healthy individuals [4]. It is an opportunistic fungus, which can cause superficial infections of the esophageal, oral, and vaginal mucosa, systemic infections [5], and represents 50% of iatrogenic fungal infections [6].

Based on the clinical condition and characteristics of each patient, the doctor will determine the therapeutic approach and indicate the best antifungal drug [7]. Among the classes of antifungals, there are the azoles [8] which can be classified into: 1) Imidazoles such as butoconazole, clotrimazole, miconazole and ketoconazole; 2) Triazoles such as itraconazole and fluconazole.

Significant hypersensitivity, toxicity, and other reactions may be observed limiting the use of these drugs [9]. Additionally, clinical complications may happen caused by multidrug-resistant strains of *Candida* spp. [10].

Several proposals have been tested to decrease drug toxicity, such as the internalization in lipid biotechnological preparations called liposomes, which increase the concentration of administered drugs without increasing their side effects [11], [12]. However, this biotechnological solution has considerably increased treatment costs, which makes treatment unfeasible in low-resource countries [13]. Thus, searching for alternatives to sensitize the pathogen to the drug seems feasible.

In this context, ultrasonic waves cause a series of phenomena, including acoustic cavitation, which increases the permeability of the cell wall, increasing the uptake of antifungals [14]. Therefore, this work intended to evaluate the effect in vitro of the therapeutic ultrasound (TUS) associated with fluconazole (FLU) on *C. albicans*.

2. MATERIAL AND METHODS

2.1 Strains and cultivation of *C. albicans*

In this study, a standard strain of *Candida albicans* (ATCC 10231, Laborclin®) was used. The experiments were conducted at the Phytopathology Laboratory at the Federal University of Acre (UFAC) and the Microbiology Laboratory at the Estácio Meta University Center in Rio Branco City, State of Acre, Brazil. *C. albicans* was seeded by exhaustion on Sabouraud Dextrose Agar (ASD) and incubated for 24 h at 37 ºC. Two colonies were selected and inoculated in 100 mL of Sabouraud Dextrose broth and incubated at 37 °C for 24 h. Then, fungal suspensions containing cells at a concentration of 1.5 x 10^8 CFU/mL were prepared using McFarland scale number 0.5. This method is an adaptation of the technique used by [15].

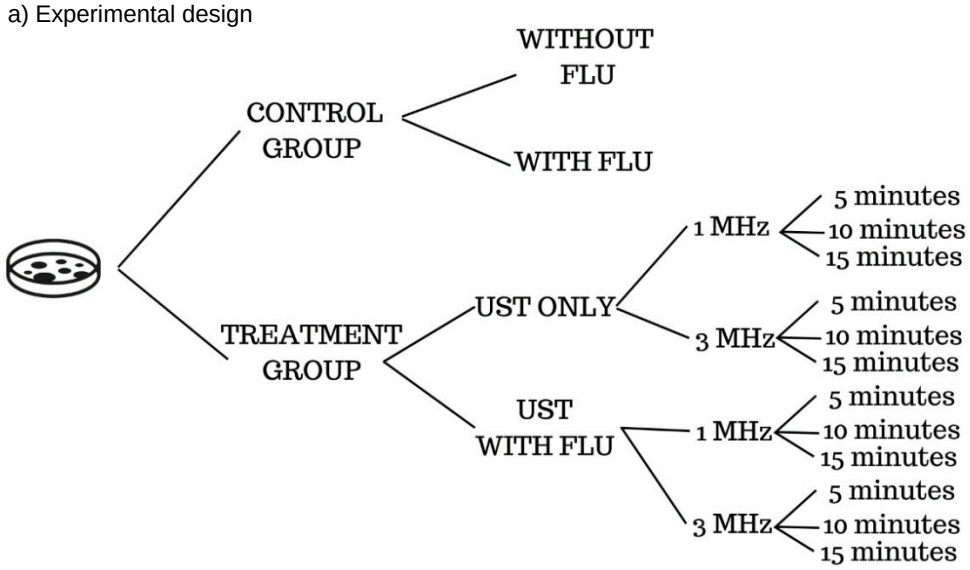
2.2 Fluconazole antifungal concentration

The Fluconazole (Belfar®) concentration used in the present study followed the Minimum Inhibitory Concentration capable of inhibiting 50% (MIC50) of the growth of C. albicans when compared to growth without an antifungal, which according to Castro et al. (2016), is 12 µg/mL. Fluconazole powder was weighted and then added to Sabouraud Dextrose Broth.

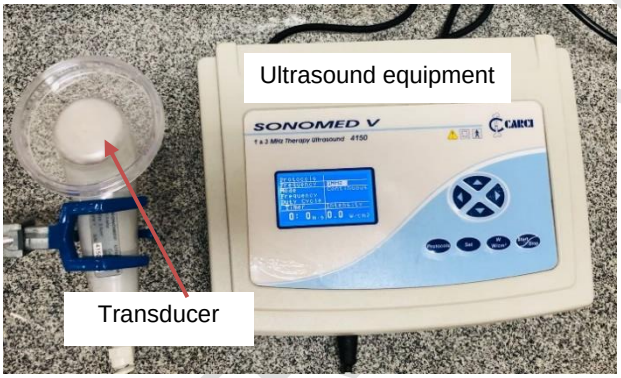
2.3 Exposure of suspensions to ultrasound

An aliquot of 1 mL of strain suspension was centrifuged at 4,000 rpm for 10 minutes. Then the supernatant was discarded, and the cell pellet was resuspended in 1 ml of physiological saline medium. The suspensions were then transferred to acrylic Petri dishes (plates) containing Sabouraud Dextrose Broth and sealed with waterproof tape. The plates were divided into Control Groups (CG, with no ultrasound treatment, with and without Fluconazole) and Treatment Groups submitted to ultrasound (UST only), and ultrasound combined with Fluconazole (UST + FLU), all experiment was developed in triplicate (Fig 1a). A conventional physiotherapeutic ultrasound (Fig 1b) device was used (Sonomed V model CARCI, São Paulo, Brazil). The intensity applied was 1.0 W/cm^2 at frequencies of 1 MHz and 3 MHz, at continuous mode. The application times were 5, 10, and 15 minutes. A water-based coupling gel was employed between the unfocused transducer and the plate to reduce their acoustic impedance mismatch (Fig 1c). Circular movements were performed with the plate which remained in direct contact with the transducer throughout the application (Fig 1c).

a) Experimental design



b) Ultrasound equipment



c) Plate with culture medium

Fig 1. a) Experimental design of groups (Canva). b) Ultrasound equipment (model Sonomed V, CARCI, frequencies 1 MHz and 3 MHz), with transducer and plate without culture medium with water-based coupling gel. c) Application of ultrasound in liquid culture medium (Sabouraud Dextrose broth), Source: Personal archive.

2.4 Cell Viability Assay

Immediately after the ultrasound treatment, 100 µL of the planktonic suspensions were removed from each plate and immediately seeded by the drip technique and spread with the aid of a Drigalski loop on Sabouraud Dextrose agar and incubated for 24 h at 37 °C to determine Colony Forming Units. The results of each triplicate experiment were shown using average and standard deviation. The survival rate of *C. albicans* was calculated as a percentage of the activity of the control group, which was defined as 100%.

2.5 Temperature Analysis

The thermal images were obtained using an infrared camera FLIR E6 (Flir Systems Inc., Wilconville, USA), with the camera lens positioned between 20 to 40 cm from the surface of the plate. The temperatures were analyzed with the help of the Flir Tools ® software.

2.6 Statistical Analysis

All data were tabulated and analyzed with the JAMOVI (https://www.jamovi.org/ ®) free software and displayed as tables and graphs. The statistical significance between the mean values of the groups (Test and Controls) was verified with a Two-Way ANOVA with Bonferroni's posterior test, at 95% confidence interval. To better compare the effects observed in the different conditions of exposure time, ultrasound frequency, and species tested, data were normalized by the respective controls, represented as Relative Growth, which was based on Equation (1):

$$Relative\ Growth = \frac{CFU\ counting\ for\ treated\ subjects}{CFU\ counting\ for\ the\ negative\ control} x100, \quad (1)$$

where CFU stands for Colony Forming Unit.

3. RESULTS

3.1 Control

In Table 1 the antifungal effect of ultrasound with or without Fluconazole is presented. The negative control without fluconazole had a CFU of 382.7 ± 15.0. The positive control with fluconazole had a CFU of 377.7 ± 23.4. No statistical difference was observed between the mean value of the controls (p = 0.779).

3.2 Ultrasound Treatment Without Fluconazole

The application of UST at a frequency of 1 MHz and intensity of 1.0 W/cm^2 with an application time of 5 minutes in the absence of fluconazole showed an average colony formation of 238.3 ± 38.7 with no statistical difference to the control groups (p<0.135). However, for the application time of 10 minutes, the average was 407.0 ± 129.4, and for 15 minutes of ultrasound, the average was 470.7 ± 23.5. This fact suggests an apparent increase in the CFU when the application time is longer than 5 minutes (Fig. 2a and Table 1).

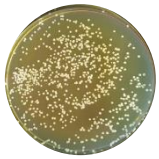
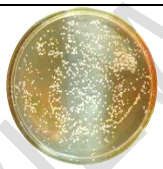
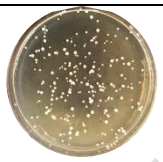
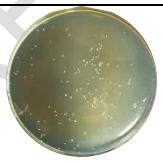
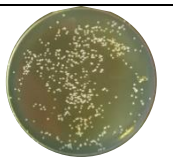
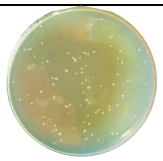
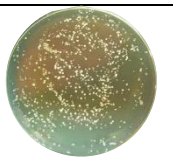
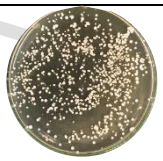
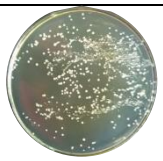
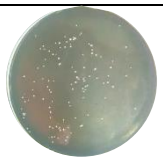
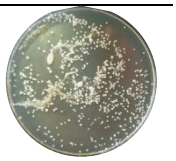
An increase in the mean number of colonies was also observed for the frequency of 3 MHz when the application time was 5 minutes (473.0 ± 33.1) in the absence of fluconazole (Fig. 2a and Table 1). Moreover, when ultrasound was applied for 15 minutes, there was a reduction in the CFU concerning the respective control group (GC), however, this reduction was not significant (382.7 ± 15,0; p=0.135).

3.3 Ultrasound Treatment with Fluconazole

When in the presence of Fluconazole, the application of UST at a frequency of 1 MHz and intensity of 1.0 W/cm^2 showed a significant reduction concerning the control (Fluconazole only) irrespective of the treatment time (p<0.0001). For the frequency of 3 MHz and the same

intensity, when the application was for 5 minutes the average formation of colonies was 262.0 ± 32.1, while for 10 minutes the average was 235.0 ± 9.5, and when for 15 minutes 299.0 ± 28.2 (Fig 2b and Table 1), with a significant reduction in the mean number of colonies when compared to the control group with Fluconazole.

Table 1. Antifungal effect across the quantification of Colony Unit Formation of *C. albicans*. Control and UST treatments. Control with and without Fluconazole. Ultrasound Treatment with 1 and 3MHz, with and without Fluconazole.

		Without Fluconazole		With Fluconazole	
Control		 382.7 ± 15.0		 377.7 ± 23.4	
UST Treatment	**Properties**	**UST only**		**UST + Fluconazole**	
	Frequency	**1MHz**	**3MHz**	**1MHz**	**3MHz**
	5 minutes	 238.3 ± 38.7	 473 ± 33.1	 133.7 ± 17.9	 262.0 ± 32.1
	10 minutes	 407 ± 129.4	 381.3 ± 20.4	 110.3 ± 19.4	 235.0 ± 9.5
	15 minutes	 470.7 ± 23.5	 259.7 ± 29.4	 122.3 ± 18.9	 299.0 ± 28.2

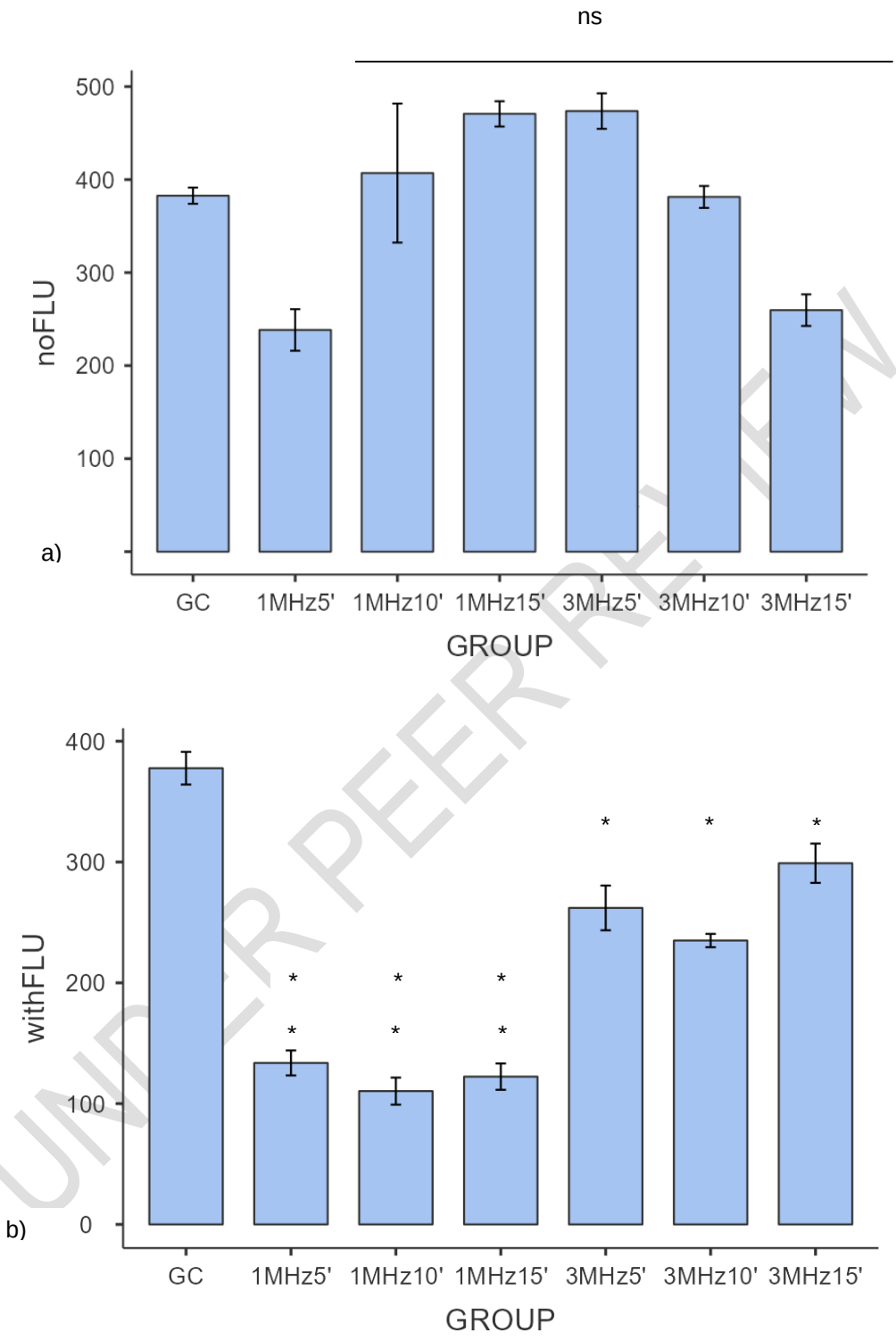
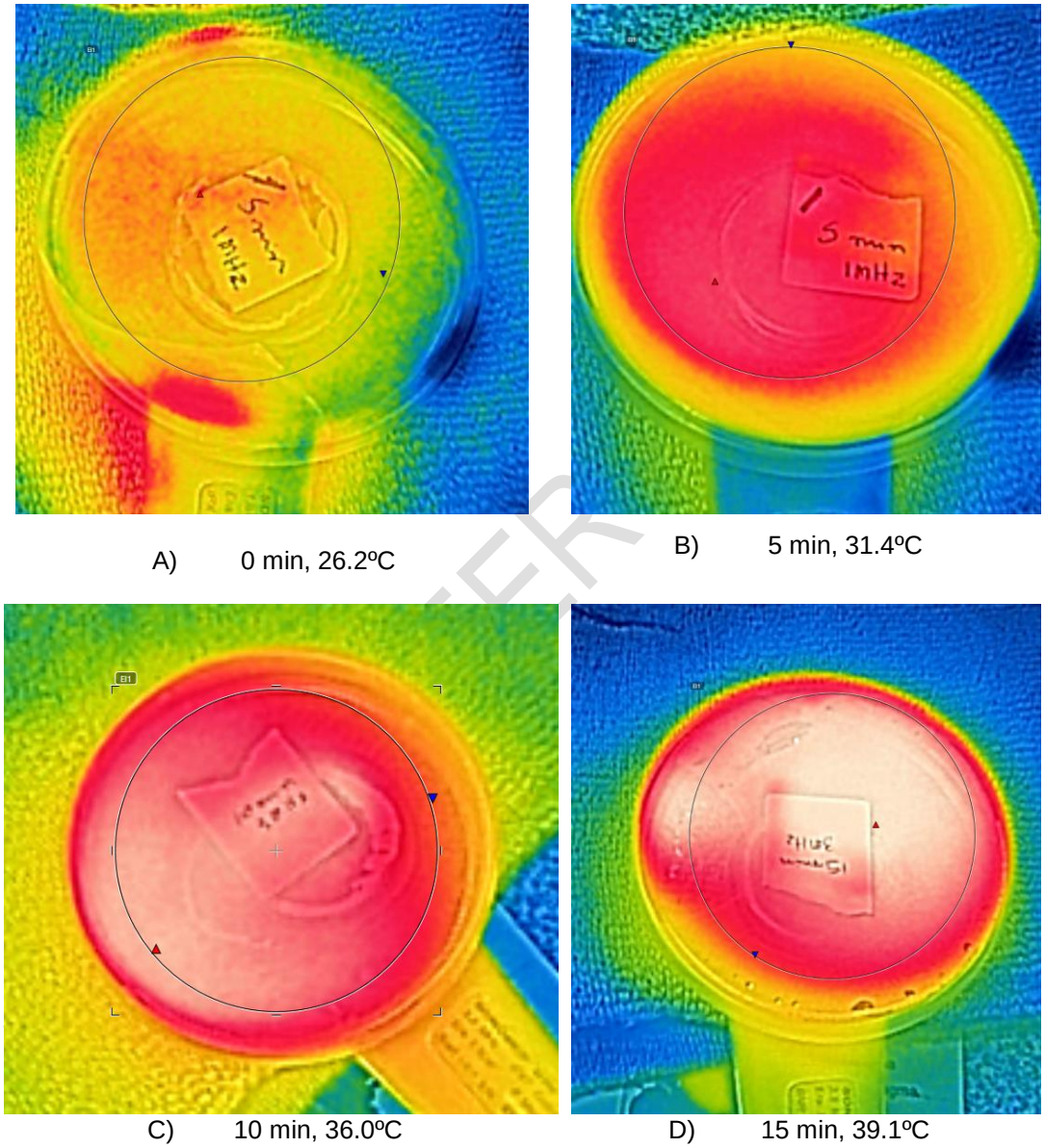


Fig 2. Mean Value of Colony Forming Units of *C. albicans*, submitted to treatments: a) Upper – No Flu and Bottom – With FLU). b) Control Flu is compared with Control without Flu in all graphs. No significant (ns) and significant (*) difference when compared to the control group (GC).

3.4 Ultrasound Treatment with Fluconazole

An increase in temperature after ultrasound application was observed in all treatments. The mean temperature was 26.2 ºC before the treatments. At the end of 5 minutes of UST application, the temperature reached 31.4 ºC, after 10 minutes, 36.0 ºC, and, after 15 minutes, 39.1 ºC. This effect was proportional to the time of the UST application, as illustrated in Fig 3.



A) 0 min, 26.2ºC B) 5 min, 31.4ºC

C) 10 min, 36.0ºC D) 15 min, 39.1ºC

Figure 3: Thermography Images of the plates. Acquisition of thermographic images of the plates: before the application of UST (Control) and after 5 minutes, 10 minutes, and 15 minutes. These images are illustrative, and the results are the simple average of the temperature of each plate after the application of the UST.

4. DISCUSSION

There was no significant difference in the CFU number between the control with and without fluconazole group. This may be related to the fact that the drug used in the present experiment was from the first-choice group (azoles), which usually leads to fungal resistance [18].

In some treatments at both frequencies, an increase in the average value of Colony Forming Units was observed. According to Rapoport et al. (1999), the application of ultrasound causes transient disturbance in the phospholipid bilayers, promoting an increase in the permeability effect, which enhances the entry of nutrients. However, this disturbance in the phospholipid bilayers can be restored after insonation, which explains some treatments remaining with their CFU averages close to those of CG [19].
It was evident that UST at a frequency of 3 MHz and intensity of 1.0 W/cm^2 applied for 15 minutes reduces the CFU value when compared to the control. Yang et al. (2018) obtained a reduction in CFU when the intensity of ultrasonic irradiation was increased, indicating that physical injury of microorganisms is associated with sound intensity [15].

In the presence of Fluconazole, both frequencies for all application times inhibited the growth of *C. albicans* colonies. A comparison of the frequencies of 1 and 3 MHz shows a difference between them for all application times of UST with an advantage for the frequency of 1 MHz.
The inhibition obtained in the treatments (UST+FLU) corroborates the results obtained by Yang et al. [15] (number of references) who used low-frequency and low-intensity ultrasound and observed that the viability of *C. albicans* did not decrease when the intensity was less than 0.3 W/cm^2 and the irradiation time was less than 15 minutes. However, when the intensity increased to 0.6 W/cm^2 or the treatment time was increased to 30 minutes, the viability of C. albicans significantly decreased when compared to the control group. Therefore, combining intensity with application time is the key to influencing the yeast death rate. The longer the application time, the higher the yeast death rate. This rate increased even more when the treatment was associated with Amphotericin B [15]. Yang and colleagues [16] conducted an in vivo study with rabbits. They developed and characterized PLGA nanoparticles transported with AmB and when combined with intravaginal ultrasound, significant synergistic antifungal activity was observed. Furthermore, the efficient removal of C. albicans from vaginal colonization and range of inflammatory inflammation, repair of pathological damage to the vaginal mucosa, and local vaginal protective immune response were presented in their results. This experiment shows that this technique can be adapted to human application.

Also, according to Yang et al. [15], after irradiation with a frequency of 42 kHz and an intensity of 0.30 W/cm^2 for 15 min, an unequal electrical potential was observed in part of the cytoplasm of the C. albicans, with edema and formation of vacuoles [15]. The yeast morphology changed after exposure to ultrasound waves, and dispersion of cells and perforations in the cell wall occurred [15]. Su et al. (2016) observed in their studies with Bacille Calmette-Guérin, similar deleterious effects after using low-frequency, low-intensity ultrasound for 15 minutes [20].

Cai et al. [21] reviewed the literature on the synergistic effect of continuous ultrasound associated with antibiotics. They observed the treatment was more effective for the ultrasonic frequency from 35 to 70 kHz and intensity lower than 4.7 W/cm^2. They observed that only one study applied a higher frequency, which was 1.5 MHz, with treatment times of 20 min. The authors conclude that low-frequency ultrasound may aid antibiotic action on planktonic and biofilm bacteria [21]. We believe that a similar effect may have occurred for the reduction in the number of colonies in our study.

According to Rapoport et al. (1999), the disturbance in the phospholipid bilayers caused by the application of ultrasound, as mentioned previously, allows the penetration of fat-soluble

substances, promoting the increase of the drug effect, considering that a greater amount of antimicrobial agent can penetrate the fungal cell [19]. Such studies evaluating the mechanisms suggest that this phenomenon may have happened in our case too.
Thermography has been used to assess ultrasound heating of biological tissues [22]. The literature demonstrates that an increase in temperature is proportional to the application time [15], [21], [23] as happened in our experiment. It is worth noting that the ultrasound transducer used could occasionally overheat its surface, then, as a preventive measure, it was kept in motion throughout the application, given that the diameter of the transducer is smaller than the diameter of the plate.

The increase in temperature may have been produced by acoustic cavitation and/or absorption, which would help explain the rise in the effect of FLU when yeasts are subjected to UST. Literature shows that increases in Reactive Oxygen Species (ROS) concentration, including H_2O_2 and O_2, induce cell death through protein denaturation and DNA filament breaks [24]. A similar effect may have happened in our experiments.

5. CONCLUSION

The most important contribution of this study is that the association of fluconazole with ultrasound is a promising strategy for treating infections caused by C. albicans, allowing a reduced therapeutic period with greater efficacy. Further studies should be carried out to optimize the treatment protocol (ultrasonic frequencies and intensities, application time, and treatment duration) for Fluconazole associated with UST against *C. albicans* as well as studies on the cytotoxicity of the association of UST with Fluconazole.

The present investigation has some limitations. Firstly, we were not able to determine whether the association of UST modifies the cytotoxicity of Fluconazole, which could suggest a different therapeutic approach since we can assume that if there was an increase in cytotoxicity against *C. albicans*, other cells such as those of vaginal mucosa may become more sensitive to the toxic action of this drug. Secondly, investigation of this therapeutic approach associated with other antifungal drugs, such as Amphotericin B, could be useful to adequately determine the UST effect.

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