EFFECT OF A NEW SUPRAMOLECULAR HYDROGEL AGAINST CANDIDA ALBICANS BIOFILM

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ABSTRACT

In recent decades, the incidence of fungal infections has considerably increased, particularly in immune compromised patients. Generally, opportunistic pathogens as Candida species are the main cause of invasive fungal infections, and are often associated with high morbidity and mortality. Candida albicans is a major fungal pathogen of humans, which present several virulence factors, especially the ability to form biofilm. The azoles are the mainly antifungal drugs used for the treatment of C. albicans infections. However, due to the presence of extracellular matrix, the C. albicans biofilm has significantly antifungal resistance, decreasing the efficacy of these conventional therapies. For these reasons, effective alternatives are required and increase the permeabilization of biofilm in order to antimicrobials reach deeper of this biofilm. So, in this study supramolecular polymer hydrogel was used as an alternative to increase the effectiveness of azoles. As results, the incubation (24 h) of mature C. albicans biofilms with the polymer (4%) associated with fluconazole (65 µg.mL⁻¹) promoted ~ 70% inactivation of this fungus, while only the antifungal at same conditions inhibited ~ 40% of C. albicans. The control group (polymer 4%) it wasn’t capable to reduced fungi viability.

Keywords: Candida albicans; Biofilm; Antifungal drugs; Supramolecular polymer.

INTRODUCTION

Candida albicans is a major human fungal pathogen, causing a variety of infections, ranging from superficial to life-threatening. (Aebi, 1984, Al-Fattani and Douglas, 2006) Recent evidences suggest that the majority of disease produced by this pathogen is associated with...
biofilms growth. Reports have been show the formation of Candida biofilm on host tissue surfaces and several medical devices, including urinary catheters, intravenous catheters, denture materials, central nervous system prostheses, artificial heart valves, joint prostheses, contact lenses, penile implants, and so on (Ramage, et al., 2006 and Doke, et al., 2014). Is important add that patients which use these device, especially catheters, may develop occult Candidemia that increase mortality rate and consequently becomes a public health problem (Nguyen, et al., 1995, Anaissie, et al., 1998 and Mukherjee, et al., 2003).

C. albicans biofilms can be defined as communities of surface-attached cells that presents an complex and heterogeneity structure containing yeast, hyphae and psuedohyphae firmly encased within a layer of extracellular matrix (ECM) (Ramage, et al., 2005, Bruzual, et al., 2007 and Ramage, et al., 2007). The biofilm lifestyle confers resistance to antimicrobials, and prevents access by host inflammatory cells and can also alter host metabolism (Estrela, et al., 2009 and de Melo, et al., 2013). In addition, due to surface- induced gene expression the biofilm cells exhibit altered phenotype if compared to the planktonic cells (Costerton, et al., 1999).

Several authors have demonstrated the C. albicans biofilms resistance to azole drugs, especially fluconazole (Baillie and Douglas, 1998, Doke, et al., 2014) and the toxic side effects of this drug limit the use of high concentrations (Shinde, et al., 2013 and Doke, et al., 2014).

Currently, wide ranges of anti-biofilm strategies have emerged, their focus include increase the permeabilization of biofilm in order to antimicrobials can penetrate deeper of this microbial community (Estrela, et al., 2009). A number of studies have reported the use of polymeric materials with significant antibiotic activity, for instance, low-molecular chitosan hydrogel (Silva-Dias, et al., 2014), chitosan derivates (Tan, et al., 2017) and polymethacrylates (Qu, et al., 2016). Here we have been used the concepts of supramolecular chemistry to designing an attractive synthesis method for preparation of a supramolecular hydrogel which can disrupt the ECM that is considerate a principal resistance mechanism of biofilms, once hamper the antimicrobial penetration.

The simplicity of their synthesis, their availability from renewable resources and the low cost of raw ingredients (fatty acids and meglumine) bode well for future applications. In our synthetic approach, we used the process of self-assembly in two levels of complexity to spontaneously fabricate the hydrogel. In first level, supra- amphiphilic building blocks were
constructed reacting the aminocarbohydrate meglumine, (2R,3R,4R,5S)-6-Methylaminohexane-1,2,3,4,5-pentol, and stearic acid (acid octadecanoic) via acid-base reaction leading to the formation of a ion pair (Cassimiro, 2012, Ferreira, et al., 2016). In contrast of conventional amphiphiles, in supra-amphiphiles the alkil chain and headgroup are attached by noncovalent bonds. In the second level, the supra-amphiphiles spontaneously undergo supramolecular polymerization via noncovalent bonds (hydrophobic interactions and hydrogen bonds) in low concentrations (~4% w/w) by heat treatment in aqueous media. Due the reversibility of hydrogen bonds, the hydrogel behaves like viscoelastic liquid when heated to 50°C and completely recovers its structure at ambient temperature. This behavior allows incorporating the antimicrobials easily.

So, the aim of this study was evaluated the effectiveness of a supramolecular polymer together with the antifungal fluconazole against C. albicans biofilm.

**MATERIAL AND METHODS**

**Synthesis of meglumine-stearate (MEG-SA) supra-amphiphiles**

Equimolar amounts corresponding of octadecanoic acid (stearic acid or SA) was reacted with meglumine (MEG) respectively in a medium composed of ethylacetate/methanol (1:1 v/v). The reaction was maintained under constant stirring for about 30 minutes at room temperature. MEG-SA precipitated as a white product that was subsequently centrifuged, washed with portions of cold ethyl acetate/methanol (1:1 v/v) and dried under nitrogen flow.

**Preparation of the supramolecular hydrogel**

The MEG-SA/water hydrogel, 4% (w/w), were prepared by mixtures of supra-amphiphile MEG-SA with purified H2O. Homogenization then occurs in the ultrasound bath at 80°C for 5 min and then cooled to room temperature.

**Candida albicans strain and culture condition**

C. albicans (ATCC 90028) was grown aerobically in Sabouraud dextrose (DifcoTM) at 37°C and 80 rpm, until logarithm phase. The yeast was harvested by centrifugation (5 min, 4000 rpm) and resuspended in sterile phosphate buffered saline (PBS) at pH 7.0 to final concentration of $1 \times 10^7$ cell.mL$^{-1}$.

**Biofilm formation**
Biofilm were formed on 96-well tissue culture plates. Hundred microliters of standardized cell suspension (1 × 10^7 cells mL^-1) and incubated for 90 min at 37°C (“adhesion phase”). Wells were gently washed with PBS to remove nonadherent cells. Subsequently, disks were immersed in Sabouraud dextrose medium and incubated for 48 h at 37°C on a rocker (“biofilm mature”).

**C. albicans biofilm sensitivity test to azoles in presence of EM hydrogel (EMH)**

After growth of the *C. albicans* biofilm, antifungal solutions were added at different concentrations (0-130 µg.mL^-1) in the presence or absence of the hydrogel (4%) as described below.

- Ketoconazole (0 – 130 µg.mL^-1) + PBS
- Ketoconazole (0 – 130 µg.mL^-1) + EM hydrogel 4%
- Fluconazole (0 – 130 µg.mL^-1) + PBS
- Fluconazole (0 – 130 µg.mL^-1) + EM hydrogel 4%

Plates were maintained at 37°C for different incubation times (24 and 48 h). Thereafter, the antifungal solutions were removed and the wells washed with PBS twice. Biofilms inhibition were quantified using a tetrazolium XTT [2,3-bis(2-methoxy- 4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay as described previously by Chandra and collaborators (Chandra, *et al.*, 2001).

All experiments were performed on replicates with their respective controls.

**Statistics**

Values are given as means and standard errors of four separate experiments. P-values of less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

As mentioned above the main of this study was applying a hydrogel to increase the effect of azoles against the *C. albicans* biofilm. Figure 1 shows the survival index (SI) of *C. albicans* biofilm after incubation with azoles in the absence and presence of the hydrogel.
Figure 1: Survival index of *C. albicans* biofilm after 24 (A) and 48 h (B) of incubation with different concentrations of ketoconazole (square) and fluconazole (circle), in the absence (black) and presence of EMH (white).

In general, the association of the antifungals with hydrogel promoted a significant inhibition of *C. albicans* biofilm, which was proportional to the increase ofazole concentration and time incubation. In addition, as has been reported in other studies, the fluconazole was more effectiveness than ketoconazole (Henry, *et al.*, 1999 and Perumal, *et al.*, 2007). After 24 h of incubation, the fluconazole (130 µg mL⁻¹) was capable to reduce around 74% of the fungi biofilm while ketoconazole (130 µg mL⁻¹) reduced ~54%. The increases of time incubation to 48 h allowed obtain the same inhibition of *C. albicans* within half of theazole concentration (65 µg mL⁻¹) in the 24 h. These results are important once highest concentration of drugs may cause a several adverse effects (De Wit, *et al.*, 1989, Grant and Clissold, 1990, Laine, *et al.*, 1992, Henry, *et al.*, 1999, Dismukes, 2000). In the other hand, only the effect of azoles was not capable to complete inhibit *C. albicans* biofilm and this generally cause a reinfection (Douglas, 2003, Garcia-Sanchez, *et al.*, 2004). The azoles resistance of *C. albicans* biofilm may be explained once these antifungals are not efficient to fully disrupt the ECM. The ECM has an extracellular polymeric substance (EPS) that acts as an adsorbent by reducing the amount of antifungal available to reach the deeper cells into biofilm structure (Chandra, *et al.*, 2001, Davies, 2003).

To convert this problem we used a hydrogel that can flow the azoles through the ECM and increase the *C. albicans* biofilm. After 48 hour of incubation the association of EMH-ketoconazole (130 µg mL⁻¹) and EMH-fluconazole (130 µg mL⁻¹) promoted a decrease of 91% and 96% in the biofilm SI, respectively. Is important highlight that only the EMH do not induce an inhibition of cells, however enables decrease the SI of *C. albicans* using less
azoles concentration e.g. EHM- fluconazole (32.5 µg.mL⁻¹) reduced ~85% of fungal cell and EHM-ketoconazole at same conditions reduced ~80% (Figure 1B). The mechanism action of EMH potentiates the antifungal effect has not been determined yet, but we may suggest that hydrogel increase the permeability of C. albicans biofilm promoting the diffusion of azoles over EPS. Probably this occurs due the chemical compatibility of the amphiphilic hydrogel with a heterogeneous composition of the ECM (Li, et al., 2013).

CONCLUSION

The necessity of new strategies to overcome the biofilm drugs resistance is already reported by several authors and this study shows that the association of antifungals and hydrogel may be a solution. Highlighting which this polymer does not cause any inhibition to biofilm but when combined with azole reduce almost 100% C. albicans biofilm SI, we may conclude that the hydrogel increases the permeabilization of antifungals allowing disrupt the ECM.

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REFERENCES

6. Cassimiro, M.; Ribeiro, CA; Crespi, MS and Boralle N (2012) “Structural aspects,


11. de Melo, WC; Avci, P; de Oliveira, MN; Gupta, A; Vecchio, D; Sadasivam, M; Chandran, R; Huang, YY; Yin, R; Peruzzi, LR; Tegos, GP; Peruzzi, JR; Dai, T and Hamblin, MR (2013) “Photodynamic inactivation of biofilm: taking a lightly colored approach to stubborn infection”. *Expert Rev Anti Infect Ther* 11: 669-693.


17. Ferreira, LM; Kurokawa, SS; Alonso, JD; Cassimiro, DL; Souza, AL; Fonseca, M; Sarmento, VH; Regasini, LO and Ribeiro, CA (2016) “Structural and Thermal Behavior of Meglumine-Based Supra-Amphiphiles in Bulk and Assembled in Water”. *Langmuir* 32: 11878-11887.

18. Garcia-Sanchez, S; Aubert, S; Iraqui, I; Janbon, G; Ghigo, JM and d'Enfert, C (2004)


22. Li, Y; Fukushima, K; Coady, DJ; Engler, AC; Liu, S; Huang, Y; Cho, JS; Guo, Y; Miller, LS; Tan, JP; Ee, PL; Fan, W; Yang, YY and Hedrick, JL (2013) “Broad-spectrum antimicrobial and biofilm- disrupting hydrogels: stereocomplex-driven supramolecular assemblies”. *Angew Chem Int Ed Engl* 52: 674-678.


