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Anti-*Candida* Activity of a Chitosan Hydrogel: Mechanism of Action and Cytotoxicity Profile

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Key Words
Chitosan hydrogel · Anticandida activity · Biodegradable

Abstract
Candida spp. are common causative agents of mucocutaneous infections. New therapeutic antifungal drugs are needed to treat chronic disease as these are frequently clinically resistant to azols. Chitosan, among other possible vehicles for active compounds, shows an added value as it appears to have intrinsic antimicrobial properties. The aim of the present study was to evaluate the anti-*Candida* activity of a medium-molecular-weight chitosan hydrogel (CH), to clarify its possible mechanism of action and to evaluate its cytotoxicity on human fibroblasts. CH antifungal activity was assessed according to CLSI reference M27-A3 protocol; its mechanism of action was investigated by flow cytometry, and its cytotoxicity was studied by MTT assay. CH demonstrated a full inhibition of *C. tropicalis*, *C. krusei*, *C. guilliermondii* and *C. parapsilosis* growth while impairing *C. albicans* and *C. glabrata* viability. Flow cytometry tests showed that CH acts by inducing primary lesion of the cytoplasmic membrane. However, CH showed no cytotoxic effect upon human fibroblasts cells. Resistant strains will require new therapeutic approaches. Chitosan being a good carrier and having itself anti-*Candida* activity seems to be a promising vehicle to be used for the treatment of mucocutaneous candidosis.

Introduction

Mucocutaneous yeast infections have increased in frequency during the last decades mostly due to the growing number of immunocompromized patients and the emergence of antifungal resistance [1]. A high percentage of such infections are caused by *Candida* spp.

Yeast infections of the vagina and vulva are among the most common gynecological conditions affecting women of all ages. Vulvovaginal candidosis (VVC) is a common clinical manifestation of *Candida* infections, with great morbidity. It affects 70–75% of women at least once in a lifetime and about 40–50% will experience a recurrence. By their 25th year of age, about half of women had at least one episode of VVC [2, 3]. Vaginal irritation, vulvar burning, pruritus and vaginal discharge are the main

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complaints associated with VVC [3]. In both sporadic VVC and recurrent vulvovaginal candidosis (RVVC), the species more frequently found are *C. albicans*, followed by *C. glabrata*, *C. tropicalis* and *C. krusei* [2–7].

VVC is usually treated very effectively with azoles. In contrast, patients with recurrent *Candida* spp. infection are difficult to manage. Most recurrences may represent vaginal relapses by persistent strains of yeast, rather than exogenous re-infections [3, 6, 7]. In fact, *Candida* spp. is considered to be a commensal microorganism of the intestinal tract. The transit from the gut through the female perineum is considered to be an important step in the process of re-inoculation of the genital tract [7].

Most gynecologists feel that the control of VVC/RVVC will require both systemic and local therapeutic approaches also involving new antifungal drugs and strategies. Recent studies revealed that the polysaccharide chitosan shows some interesting antifungal and antibacterial properties [8].

Chitosan is a natural cationic amino polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine, formed by alkaline deacetylation of chitin, a substance obtained from crustaceans, insects and fungi. The molar fraction of glucosamine residues is referred to as the degree of deacetylation [9–11].

Chitosan lacks irritant or allergic effects, has been described to have low toxicity and is considered to be biodegradable. Its degradation products are also nontoxic, nonimmunogenic and noncarcinogenic [12, 13].

The aim of the present study was to assess the anti-*Candida* activity of a medium-molecular-weight chitosan hydrogel (CH), to elucidate its possible mechanism of action and to assess its cytotoxicity on human fibroblasts in order to evaluate its potential as a therapeutic tool for mucocutaneous *Candida* infections.

Material and Methods

Chemicals and Drugs

Highly (98%) purified chitosan (average M_η = 270,000 Da, 86% deacetylated) was kindly gifted by Ceramed (Lisbon, Portugal). Amphotericin B, Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM-F12), epidermal growth factor (EGF), fibroblast growth factor (FGF), heparin, insulin, L-glutamine, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin G, phosphate-buffered saline (PBS), propidium iodide (PI), RPMI 1640 culture medium, streptomycin, and trypsin were purchased from Sigma (Sintra, Portugal). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany).

*Anti-*Candida *Chitosan Hydrogel Activity*

Yeast Strains

Candida spp. strains used corresponded to clinical isolates of *C. albicans* (n = 2), *C. krusei* (n = 2), *C. glabrata* (n = 2), *C. parapsilosis* (n = 2), *C. tropicalis* (n = 2) and *C. guilliermondii* (n = 2). In addition, strain 10231 from American Type Culture Collection (ATCC) was also used. The clinical isolates were obtained from mucocutaneous infection; all strains were isolated from patients with severe clinical conditions, some with recurrent disease and showing variable degree of resistance to fluconazol. Such isolates had been characterized to species level using API 32C (BioMérieux, Vercieux, France). The strains were kept frozen in brain-heart broth (Difco Laboratories, Detroit, Mich., USA) with 5% glycerol at –70°C until testing. For each experiment, the yeasts were subcultured twice on Sabouraud agar (Difco) to assess the purity of the culture and its viability.

*Anti-*Candida *Activity*

Chitosan was previously dissolved in acetic acid (Merck) 1% v/v solution to a final concentration of 5% w/w. Serial dilutions of the gel were then performed with sterile distilled water.

A protocol based on CLSI reference M27-A3 macromethod was developed in order to study the anti-*Candida* activity of CH [14]. Briefly, volumes ranging from 100 to 10 μl of chitosan hydrogel were dissolved in 900 μl of RPMI, previously inoculated with the strain. The relation between the hydrogel volume tested and its mass was established, allowing the definition of MIC as mass of chitosan (mg) per volume of inoculated medium (ml). MICs were read after 24 and 48 h of incubation at 37°C. For each CH concentration, yeast growth was visually compared with the control sample. Additional controls were performed with RPMI medium containing 1% acetic acid. All determinations were performed in duplicate.

Mechanism of Action

The effect of the CH on fungal cell were elucidated by flow cytometry, as previously reported [15]. In summary, after incubating 10^6 cells/ml for 1 h at 37°C with CH at half MIC, MIC and double MIC, the cells were stained with propidium iodide (PI, Sigma), 1 μg/ml, protected from light, at room temperature, during 15 min. PI is a fluorescent probe that selectively stains cells with severe membrane damage. Following the staining step, the cells were analyzed on a FACSCalibur cytometer (BD Biosciences, Sydney, N.S.W., Australia) at FL3 (620 nm red).

Nontreated and non-PI-stained cells were used to determine autofluorescence; nontreated and stained cells were used as viability control, and yeast cells treated with 70% ethanol for 10 min were used as a death control.

After treatment with CH for 60 min, cells were plated on agar medium for evaluation of the number of colony-forming units (CFU).

For kinetic studies, yeast cells were incubated during 5, 10, 15 and 30 min with CH at MIC and stained with PI using the protocol described above.

Cytotoxic Effect

Fibroblast Cell Culture

Fibroblast cells from human skin were obtained as reported in the literature [16]. The isolated cells were plated in T-flasks containing 25 cm^3 DMEM F-12 medium (1:1 v/v) supplemented with

heat-inactivated FBS (5% v/v), EGF (5 μg/ml), FGF (0.5 ng/ml), heparin (2 μg/ml), insulin (5 μg/ml), L-glutamine (2 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml).

After 2 h, the nonadherent cells were washed out. Cells were kept in culture at 37°C, in a humidified atmosphere with 5% CO_2. After achieving confluent growth, cells were subcultured for 5 min in 0.18% trypsin (1:250) and 5 mM EDTA. To stop the trypsinization process fresh culture medium was added to the free cells.

To examine cell proliferation in the presence of CH, fibroblast cells were cultured in 12-well plates with 1×10^5 cells/ml for 24 h. Cell growth was monitored using an Olympus CX41 inverted light microscope equipped with an Olympus SP-500 UZ digital camera.

Determination of Chitosan Hydrogel Cytotoxicity by MTT Assay

CH at different concentrations was diluted in cell culture medium and incubated at 37°C for 24 h without agitation (n = 6) [17]. Afterwards, it was added to a 96-well plate containing the human fibroblast cells (40,000 cells per well) and incubated for 24 h at 37°C, in a 5% CO_2 humidified atmosphere. The culture medium was discarded and the mitochondrial redox activity of fibroblast cells was assessed through the reduction of the MTT. Briefly, 50 μl of MTT (5 mg/ml PBS) were added to each well and incubated for 4 h at 37°C, under a 5% CO_2 humidified atmosphere. Afterwards, the culture medium was aspirated and fibroblasts were treated with 50 μl of isopropanol/HCl (0.04 N) for 90 min. Then the absorbance was read at 570 nm using a Biorad Microplate Reader Benchmark.

Wells containing cells in culture medium without CH were used as negative control; ethanol 96% was added to wells containing cells, as a positive control.

Data Analyses

Statistical analysis was performed using one-way ANOVA with Dunnett's post-hoc test. Computations were performed using a MYSTAT 12 statistical package (Systat Software, a subsidiary of Cranes Software International, Ltd.).

Results

*Anti-*Candida *Activity of Chitosan Hydrogel*

The determination of MIC values showed that CH was active against the *C. tropicalis*, *C. krusei*, *C. guilliermondii* and *C. parapsilosis* tested strains. However, at the maximal concentration used (5 mg/ml), CH was unable to completely inhibit the growth of *C. albicans* and *C. glabrata* (table 1). The growth of the tested strains was not influenced by the presence of acetic acid. The results from three independent experiments were invariably concordant.

Table 1. *Candida* strains tested, susceptibility to classical antifungals and MIC results for CH used

Yeast	Antifungal susceptibility MIC μg/ml		Chitosan hydrogel MIC mg/ml
	fluconazole	amphotericin B	
C. albicans ATCC 10231	1	0.5	>5.0
C. albicans 28	>64	0.5	>5.0
C. albicans 30	8	0.5	>5.0
C. glabrata H16	1	0.25	>5.0
C. glabrata H30	>64	0.5	>5.0
C. tropicalis ARTEMIS 41	16	0.5	0.9–1.0
C. tropicalis ARTEMIS 35	>64	0.5	0.9–1.0
C. krusei OL103	>64	0.5	0.05
C. krusei OL099	>64	0.5	0.05
C. parapsilosis 011	1	0.5	0.015
C. parapsilosis 030	0.5	0.25	0.015
C. guilliermondii ARTEMIS 32	4	0.5	0.003
C. guilliermondii ARTEMIS 33	2	0.5	0.003

Mechanism of Action

Cytometric results revealed an evident fungicidal effect of CH after incubation during 1 h at MIC (fig. 1) due to membrane lesion. In a kinetic study conducted at MIC, 15 min were sufficient to produce an effect similar to that of 30 min, in terms of cell death (fig. 2). Interestingly, a small subset of yeast cells was not stained with PI despite being death, as confirmed by CFU assessment (fig. 3).

Although a MIC was not established for *C. albicans* and *C. glabrata* strains, a cytometric test was performed in conditions described above at the high CH concentration tested (5 mg/ml) revealing the impairment of viability in a variable extent of the yeast cell population (fig. 4).

Cytotoxicity of Chitosan Hydrogel

After incubating the human fibroblasts in presence of different concentration of CH and in its absence, viable cells were observed. In the positive control (death control), no cell adhesion or proliferation was observed, fibroblasts showed the typical spherical shape of dead cells. In addition, viable cells were observed either in the presence of different concentration of CH or in the absence of the biomaterial.

The MTT assay results showed a significant difference between cells exposed to CH (1.6 and 3.2 mg/ml) and the positive control ($p < 0.05$), after 24 h of incubation (fig. 5).

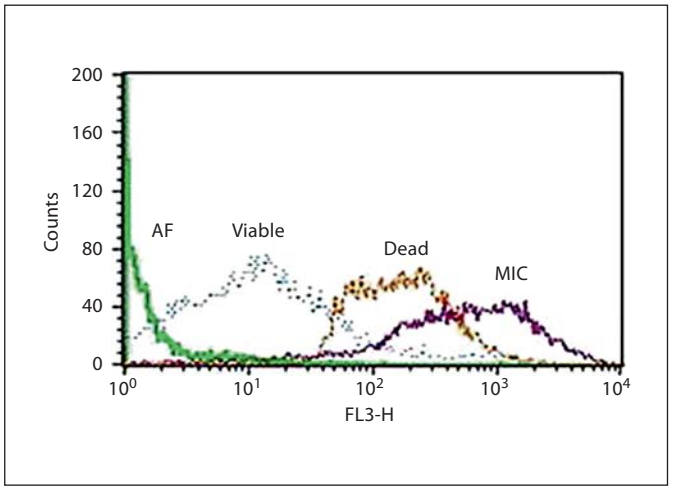

Fig. 1. Histogram representing PI-stained cells. AF = autofluorescence; viable = nontreated cells (viable control); dead = cells treated with 70 ethanol (dead control); MIC = cells treated with MIC concentration of CH during 1 h.

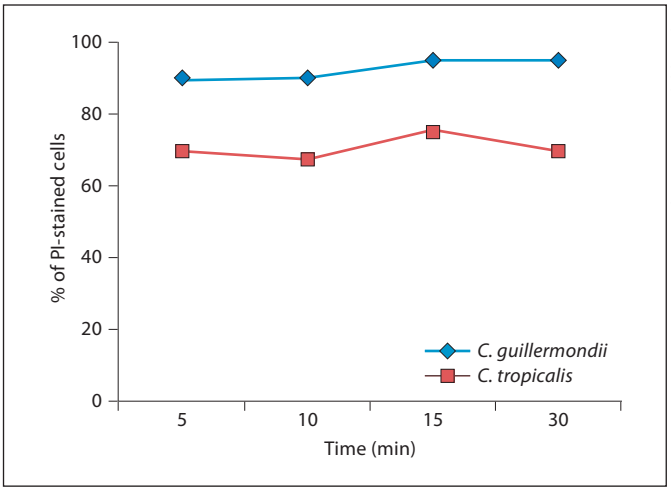

Fig. 2. Kinetic study showing the percentage (%) of PI-stained cells after treatment with MIC concentration during 5, 10, 15 and 30 min, i.e. dead cells.

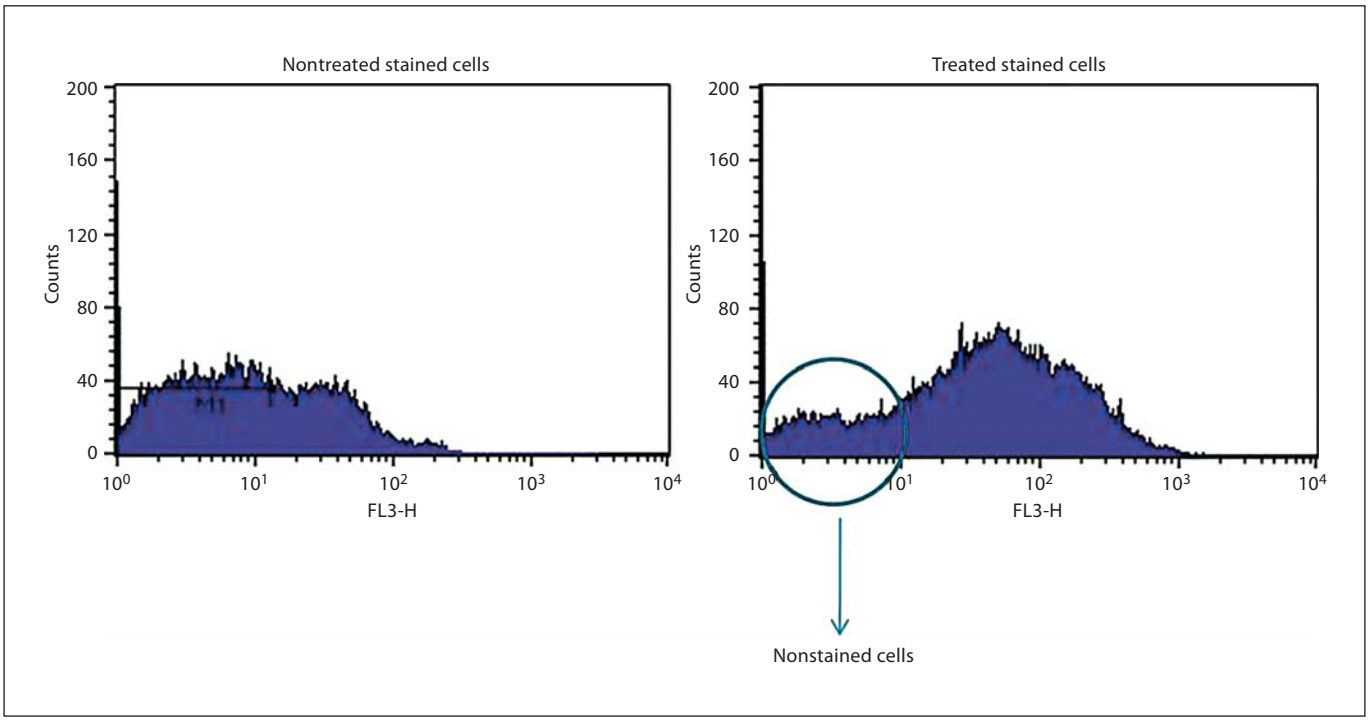

Fig. 3. Histogram showing nonstained cells after treatment with CH.

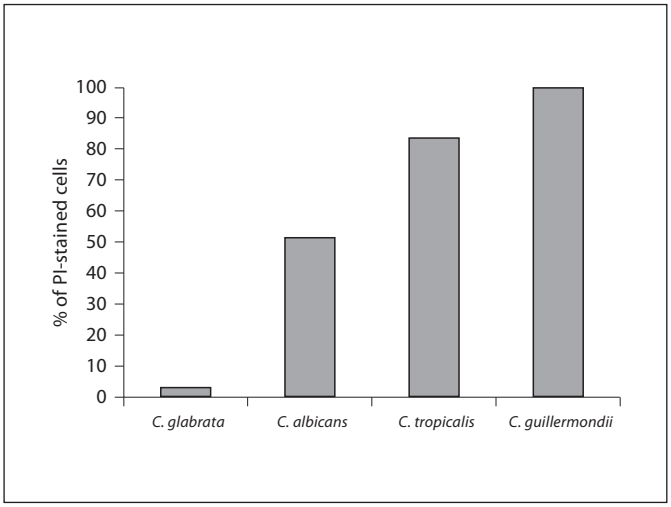

Fig. 4. Histogram showing the percentage (%) of PI-stained *C. albicans* and *C. glabrata* cells after treatment with 5 mg/ml CH for 1 h, i.e. dead cells.

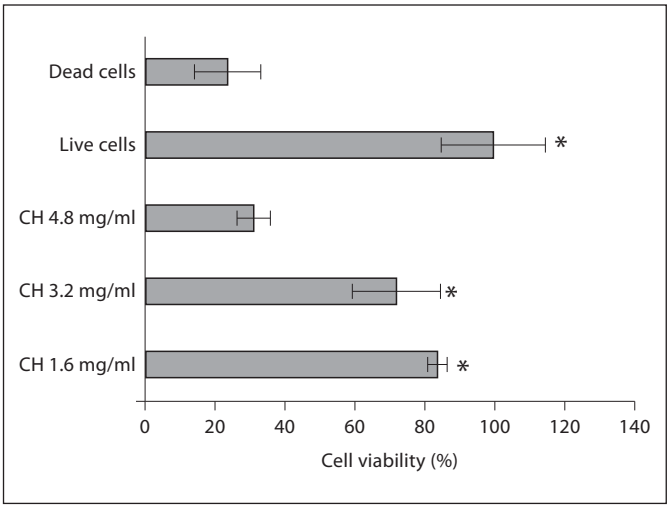

Fig. 5. Cellular activity measured by the MTT assay. Dead cells were used as positive control; living cells were used as negative control. Each result is the mean ± SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post-hoc test (* $p < 0.05$).

Discussion

In the present study we were only able to determine the MIC value for non-*albican* strains. Regarding *C. albicans* and *C. glabrata*, the complete inhibition of the growth was not observed at the tested concentrations, suggesting that such species may have specific membrane characteristics that confer resistance to the tested chitosan. Nevertheless, the cytometric analysis revealed that for *C. albicans*, 50% of the cells were killed at the highest CH concentration tested; *C. glabrata* revealed a more resistant pattern.

Our results are in accordance with Zakrzewska et al. [18] showing that chitosan affects growth of *C. albicans* and *C. glabrata* in a dose-dependent manner, resulting in a higher inhibitory effect upon *C. albicans* than *C. glabrata*. No fungicidal effect was claimed for the compound tested, regarding those 2 species, and no MIC value was also published.

The results from CH anti-*Candida* activity indicate that there is no correlation between MIC of CH and MIC of classical antifungal, suggesting that the mechanism of CH action is distinct.

The cytometric results demonstrated an evident fungicidal effect after incubation during 1 h with MIC values. As PI quickly entered the cell, we may conclude that CH induced a primary lesion of the cytoplasmic membrane.

The formation by CH of a layer involving the outer surface of the yeast is probable to occur since a subset of cells, although not stained with PI, was shown to be dead as confirmed by culture; such an effect could impair the penetration of the fluorescent marker into the dead yeast cell.

Chitosan antifungal activity has been explained as the result of a binding reaction between protonated chitosan amino groups with negatively charged cell surface molecules [13]. This theory is supported by the findings of Seyfarth et al. [8] that reported that N-acetyl-D-glucosamine chitosan was unable to inhibit *C. albicans* and *C. krusei* growth and slightly reduced the growth of *C. glabrata*. This effect contrasted with that of other chitosan derivatives that, as the polysaccharide included in our study, by having a free amino group demonstrated a good anti-*Candida* effect. Regarding the proposed mechanism of action, high-molecular-weight chitosans are expected to demonstrate better anti-*Candida* activity as they possess more chemical groups available for such reactions.

In order to evaluate the safety of CH for biomedical applications, its cytocompatibility was evaluated through in vitro studies. Different assays are currently available to characterize biomaterial cytocompatibility. MTT assay was chosen, since it is a fast and effective method to evaluate cellular viability. In recent years, it has been frequently used as a preliminary screen test to evaluate the in vitro cytotoxicity of biomaterials.

The MTT assay showed that mitochondrial function and the cell proliferation profiles differed significantly between cells exposed to CH and to ethanol (death control), after 24 h of incubation. In addition, CH was able to promote fibroblast adhesion and proliferation. This result is in accordance with others described in the literature [19]. Our results demonstrate that CH did not affect cell viability, supporting the assumption of such formulations being devoided of acute cytotoxic effect in vivo.

In conclusion, although the potency of anti-*Candida* activity of CH differs according to the *Candida* species evaluated, its antifungal effect has been demonstrated. This hydrogel was able to completely inhibit the growth of *C. tropicalis*, *C. krusei*, *C. guilliermondii* and *C. parapsilosis* due to a fungicidal effect. In contrast, no MIC was established for *C. albicans* and *C. glabrata*. Nevertheless, its toxic effect, especially upon *C. albicans*, was shown, resulting in cell death in a significant percentage of yeasts.

Flow cytometry results showed that CH anti-*Candida* activity results from lesion of the cytoplasmic membrane of fungal cell. In vitro culture assays showed that the CH was able to promote cell adhesion and proliferation.

Attending to its nontoxic and muco-/bioadhesive properties, chitosan polymers have been proposed as drug delivery system. Based on the anti-*Candida* activity and on its ability to improve adhesion between drug formulation and the mucosal tissue, chitosan seems to be promising as a vehicle to be used for drug delivery system in clinical cases of mucocutaneous infections. It is expected that chitosan, as being active as an antimicrobial, will enhance the action of main antifungal drugs to whom it will serve as carrier.

This work reports the activity of a single medium-molecular-weight chitosan. Studies with other chitosans with different molecular weights will follow, as well as in vitro studies regarding the activity of these compounds on *Candida* biofilms.

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