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# Microbicidal activity of neutrophils is inhibited by isolates from recurrent vaginal candidiasis (RVVC) caused by *Candida albicans* through fungal thioredoxin reductase

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Vulvovaginal candidiasis (VVC) is characterized by an infection of the vulva and vagina, mainly caused by *Candida albicans*, a commensal microorganism that inhabits the vaginal, digestive, and respiratory mucosae. Vulvovaginal candidiasis affects approximately 75% of women, and 5% develop the recurrent form (RVVC). The aim of the present study was to evaluate whether neutrophils microbicidal response is triggered when activated with RVVC isolates caused by *C. albicans*. Our results showed that RVVC isolates induced neutrophil migration but significantly decrease the microbicidal activity of neutrophils, compared with VVC and ASS isolates. The microbicidal activity of neutrophils is highly dependent on the production of reactive oxygen species/reactive nitrogen species (ROS/RNS). However, this isolate induced detoxification of ROS/RNS produced by neutrophils, reflected by the high level of thiol groups and by the oxygen consumption. Therefore, RVVC isolates induced biochemical changes in the inflammatory response triggered by neutrophils, and these effects were mainly related to the detoxification of ROS/RNS through the thioredoxin reductase (TR), a key antioxidant enzyme in fungi. This might be one of the resistance mechanisms triggered by RVVC caused by *C. albicans*.

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## 1. Introduction

Vulvovaginal candidiasis (VVC) is an infection that affects approximately 75% of women during their lifetime [1]. Of these, 40–50% will have new outbreaks, and 5% will develop the recurrent form (RVVC) [2]. This is one of the most common diagnoses in gynecology. Its high incidence makes it the second most common genital infection in the United States and Brazil. In Europe, it is the leading cause of vulvovaginitis [3], representing 20–25% of infectious vaginal discharge, preceded only by bacterial vaginosis [4].

The high incidence of RVVC might be attributable to the powerful virulence factors of *Candida albicans* [2] and the various immune responses triggered in different hosts [5]. Therefore, studies that investigate both the yeast and innate immune responses in the host may be especially informative because RVVC induces host immunoregulation [6,7]. However, little is known concerning the role of innate response in VVC and RVVC infection, as far as we know, there have been no studies describing the activity of neutrophils in women with VVC or RVVC infection. The literature has well documented studies only in the *in vitro* activity of neutrophils against *Candida* species [8]. Evidences support the concept that

*Abbreviations:* ASS, asymptomatic; ATCC, reference strain; $CaCl_2$, calcium chloride; DHR 123, dihydrorhodamine 123; DMSO, dimethylsulfoxide; DTNB, 5-5′-dithio-bis(2-nitrobenzoic acid); fMLP, *N*-formyl-Met-Leu-Phe; $H_2O_2$, hydrogen peroxide; HOCl, hypochlorous acid; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; Rh123, rhodamine 123; ROS, reactive oxygen species; RNS, reactive nitrogen species; RVVC, recurrent vulvovaginal candidiasis; SDA, Sabouraud dextrose agar; TMB, 3,3′,5,5′-tetramethyl benzidine; TR, thioredoxin reductase; VVC, vulvovaginal candidiasis.

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the pathogenesis of RVVC in women with no risk factors is related to depression of the local immune response, which may account for the tolerance of the vaginal mucosa to the yeast [9,10]. Thus, women with impaired immunity have been considered a high-risk group.

The present study sought to determine whether neutrophils response is triggered when activated with RVVC isolates caused by *C. albicans*. Our goal is to evaluate the microbicidal activity of neutrophils induced by RVVC compared with VVC and asymptomatic isolates (ASS) caused by *C. albicans* and the reference strain. The present results clearly demonstrated that RVVC isolates significantly decrease neutrophil microbicidal activity. We suggest that one of the resistance mechanisms of RVVC isolates of *C. albicans* might be attributable to the detoxification of reactive oxygen species/reactive nitrogen species (ROS/RNS) produced by neutrophils, through the NADPH oxidase system, induced by thioredoxin reductase (TR), a key antioxidant enzyme in fungi [11].

## 2. Materials and methods

### 2.1. Chemicals

Dextran, Histopaque, taurine, 3,3′,5,5′-tetramethyl benzidine (TMB), phorbol 12-myristate 13-acetate (PMA), *N*-formyl-Met-Leu-Phe (fMLP), dihydrorhodamine 123, catalase, RPMI-1640 medium, and 5-5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hypochlorous acid (HOCl) was prepared by diluting a concentrated commercial chlorine solution and calculating its concentration using its absorption at 292 nm (292 nm = 350 $M^{-1}$ $cm^{-1}$). PMA was dissolved in 10 μg/ml dimethylsulfoxide (DMSO). TMB solution was prepared by dissolving 10 mM TMB and 100 μM potassium iodide in 50% dimethylformamide and 50% acetic acid (400 mM). Dihydrorhodamine 123 was dissolved in 10 μg/ml DMSO. All of the experimental groups, including the controls, were tested with final DMSO concentrations of less than 10%, a concentration that was found to not affect neutrophil viability (data not shown).

### 2.2. Cell isolation

Neutrophils were isolated from peripheral venous blood obtained from different healthy donors by centrifugation over a Ficoll-Hypaque gradient (Histopaque; $d$ = 1.077 g/ml) according to Boyum et al. [12]. Cell concentration and viability, determined by trypan blue exclusion, were determined in a Neubauer chamber. The purity was estimated to be higher than 98%. Neutrophils ($2.0 \times 10^6$ cells/ml) were suspended in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 2 mM L-glutamine and used for all of the assays. The volunteers signed a consent form, this study was approved by the Committee for Ethics in Research Involving Humans at the State University of Maringá (UEM)/Paraná, Brazil (No. 293/2006).

### 2.3. Yeast collection

The present study used five clinical isolates from female vaginal secretions that belonged to the archive collection of the Laboratory of Medical Mycology, Universidade Estadual de Maringa, Brazil, and one reference strain from the American Type Culture Collection (*C. albicans* ATCC 90028). The clinical isolates of candidiasis infection were separated into groups according to symptoms presented by the patients: four isolates from patients with well defined symptomatic episodes, three of them with RVVC (RVVC1, RVVC2, and RVVC3) infection (recurrent episodes) and one with VVC infection (unique episodes). The fifth was isolated from an asymptomatic (ASS) patient, with no history of VVC [13]. All of the isolates were previously identified using CHROMagar Candida (CHROMagar, BioMerieux, Paris, France) and matrix-assisted laser-desorption/ionization time-of-flight mass spectroscopy (MALDI TOF-MS) [14,15].

*C. albicans* was grown overnight on Sabouraud dextrose agar (SDA) plates at 37 °C. The yeast was then scraped from the plates and suspended in sterile saline. The quantity of viable yeast was estimated in a Neubauer chamber. Opsonization using pooled serum from healthy donors (stored in portions at −20 °C) was achieved by incubating *C. albicans* ($2.0 \times 10^7$ yeast/ml) with 10% serum (v/v, final concentration) for 30 min at 37 °C and whirlmixing. Opsonized yeast was used for all of the assays.

### 2.4. Migration assay

Migration was evaluated in a 48-well microchamber (Neuro Probe, Cabin John, MD, USA). Separation was performed using a polycarbonate membrane with 5 μm diameter pores according to Ayres-Sander et al. [16]. The following were placed in the lower chamber: 26 μl ($2.0 \times 10^7$ yeast/ml) of different isolates of *C. albicans* (RVVC1, VVC, and ASS) and the reference strain, 10 nM fMLP as a chemotactic stimulus diluted in RPMI (i.e., positive control), or the RPMI culture medium (i.e., negative control). Neutrophils (50 μl; $1.0 \times 10^6$ cells/ml) were placed in the upper chamber. The chamber was then incubated for 1 h at 37 °C with 5% $CO_2$. The polycarbonate membrane was removed, washed, fixed, and stained (Hema 3 stain kit, Biochemical Sciences, Bridgeport, NJ, USA). The number of cells that migrated to the bottom of the membrane was counted using a 100× objective in five random fields. The results are expressed as the number of neutrophils per field.

### 2.5. Determination of hypochlorous acid

The formation of HOCl was based on the formation of taurine-chloramine that results from the reaction of HOCl with taurine as described by Dypbukt et al. [17]. Neutrophils ($2.0 \times 10^6$ cells/ml) that were activated or not activated with different isolates of *C. albicans* (RVVC1, RVVC2, RVVC3, VVC, and ASS) and the reference strain ($2.0 \times 10^7$ CFU/ml) and 400 nM PMA in 10 mM phosphate buffer (pH 7.4) that contained 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mg/ml glucose were incubated with 15 mM taurine at 37 °C with gentle agitation. The reaction was stopped after 30 min by the addition of 20 mg/ml catalase, followed by $700 \times g$ centrifugation for 10 min at 24 °C. The concentration of taurine-chloramine present in the supernatant was estimated by adding 50 μl TMB solution. The resulting blue product was detected spectrophotometrically at 655 nm using a plate reader (WaveX5 power-Biotech, USA).

### 2.6. Dihydrorhodamine 123 assay

Dihydrorhodamine 123 (DHR) is normally used to detect intracellular oxidant species production by cellular systems. The non-fluorescent probe DHR is oxidized to the fluorescent product Rh123 when it is in contact with intracellular oxidant species [18]. Total leukocytes ($2.0 \times 10^6$ cells/ml) were activated or not activated with different isolates of *C. albicans* (RVVC1, VVC, and ASS) and the reference strain ($2.0 \times 10^7$ CFU/ml) and 400 nM PMA and incubated with 50 μM DHR for 30 min, washed once with PBS, and suspended in PBS. The fluorescence of gated neutrophils was detected at FL1, with 10,000 events/gate, using a FACSCalibur flow cytometer (Becton-Dickinson, Rutherford, NJ, USA). The data were analyzed using CellQuest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA), and the results were

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