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Evaluation of Aspirin effect on *Candida Glabrata* isolates with resistance to azole compounds by real-time PCR

Abstract

Background and objective: Among the *Candida* species, *Candida glabrata (C. glabrata)* is inherently resistant to anti-mycotic agent, azole. The aim of this study was to assess the effects of Aspirin as an anti-inflammatory drug on azole-resistant *Candida glabrata in- vitro.*

Materials and methods: Five *C. glabrata* isolates were collected from patients with candidal vulvovaginitis referred to clinic. The antibiotic susceptibility test of isolates for fluconazole was performed according to the CLSI M38-A2 standard protocol. A 64µg/ml aspirin and 500ug/ml and likewise 64µg/ml fluconazole were used for the MIC detection test. The RNA of isolates was prepared according to the manufacturer's instruction. Synthesis of cDNA was conducted with the 1621K kit (Fermentase).. The RT-PCR reaction was set up for *ERG3*, *ERG6* and *ERG11* genes and the housekeeping gene Beta-actin (β-actin) was amplified as internal control.

Results: All of the isolates were resistant to fluconazole and aspirin. In the concentration of the aspirin at the 500ug/ml and the MIC and MBC were 15.62µg/ml and 31.25µg/ml, respectively. In the RT-PCR reaction, the *erg6* was expressed at the concentration of 15.62µg/ml, but no expression was detected without the aspirin exposure. However, the expression profile of *erg3* and *erg11* was not detected. However, aspirin conferred a fatal effect at the concentration of 31.25µg/ml, as no growth of the strains was observed.

Conclusion: The present study exhibited that aspirin induce the anti-fungal effect of fluconazole at high concentrations conferring a synergistic effect and fungicidal activity.

Keywords: *Candida glabrata*, Aspirin, Azole-resistance, *In vitro*

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Introduction

Candida glabrata that was previously viewed as a nonpathogenic commensal microorganism of human mucosal tissues, is the second or third nosocomial *Candida sp* and related infections (mucosa and systemic) have significantly increased due to the use of immunosuppressive agents.[1] Among the *Candida* species, *C. glabrata* is inherently resistant to azole anti mycotic agents. A common mechanism for resistance is reduction of antifungal drug via over expression of efflux pumps (Cdr1, Cdr2 and Mdr1).[2–4] Cdr1 and Cdr2 are belonged to the ABC family and ATP dependent trans- membrane proteins. Several previous studies have exhibited those modulators such as ibuprofen; FK506 (tacrolimus) and calcineurin inhibitor can revert resistance or suppress the development of resistance of *Candida spp*.[5,6] The mutations occurred in several ergo sterol biosynthesis genes including *ERG1*, *ERG3*, *ERG6*, *ERG7*, *ERG9* and *ERG11* confer the resistance to azoles.[7] The nonessential gene *ERG6* encoding an *S*-adenosylmethionine:_24 methyltransferase, undergoes mutations which can develop multiple phenotypes, including, decreased ergo sterol content, increased resistance to polyenes and increased cyclohexamide sensitivity.[8] The accumulation of *ERG3* gene products including14_-methylated sterols and 14_-methylergosta-8, 24(28)-dien-3, 6-diol play an important role in azole sensitivity, thus inactivation of the gene or mutations can confer the resistance, because of lack of enzymatic degradation. In *C. glabrata* mutation in *ERG3* does not always caused resistance to azole compounds.[9] Occurrence of point mutations in the *ERG11* gene encoding lanosterol 14a-demethylase is another mechanism of resistance.[10] The *erg11* positive selection was done on a polyene-containing medium and under aerobic conditions.[9] the expression of *ERG11* decreases in the stationary phase of growth of *C.albicans* and causes resistance of biofilms to azole drugs.[11] The non-streoidal anti-inflammatory drugs (NAID) especially Ibuprofen Diclofenac and Aspirin have been shown to have inhibitory effect on biofilm formation and decrease of drug resistance.[12] Several studies on the other hand, have shown that aspirin can effect on *Staphylococcus epidermidis* biofilms.[13] The aim of this study was to assess the effects of Aspirin as an anti-inflammatory drug on azole-resistant *C. glabrata invitro*.

Materials and methods

Fungal isolates

Five standard *C. glabrata* isolates 625, 10116, 8933, 194, 634 and 2010 were separated from patients with candidal vulvovaginitis referred to the female clinic of Dr. Shariati hospital of Tehran. The *C. glabrata* cultures were identified with conventional tests.

The minimum inhibitory concentration (MIC) test

The antibiotic susceptibility test of *C. glabrata* isolates for fluconazole and aspirin was performed according to the CLSI M38-A2 standard protocol.[14] A suspension of 5×104 concentration of each isolate was prepared. The serial dilution for MIC was done by mixing RPMI 1640 medium and drugs in sterile saline (each = 100µl) into 96 well plates and inoculated. The RPMI with and without suspension were used as the control positive and negative, respectively. The plates were incubated in 35°C for 72h and the pH was equal to 7 (repeated 5 times). For the confirmation of growth of isolates, 10µl of each well component was inoculated to the sabouraud dextrose agar plates. The

MIC and minimum fungicidal concentration (MFC) were measured considering the growth on SabDex agar and counting of colonies.

Aspirin effect on resistant isolates

Concentrations of 64µg/ml and 128µg/ml aspirin were used for the MIC detection test. Each stock was used into two different concentrations of CLSI protocol and without dilution. The alcohol and DMSO were separately used for dilution of aspirin. Next, the isolates suspension concentration was diluted 1/10. The effect of sub-inhibitory amount of 200ul volume of aspirin (64 and 128 and 256µg/ml) in forms of CLSI protocol and without dilution) and also fluconazole in volume and concentration equal to that of aspirin were measured. Moreover, in higher concentration of aspirin (500 µg/ml), the MIC and MBC of aspirin was detected. In each micro-plate, 100ul of each of drug, and RPMI was inoculated.

RNA Extraction

The RNA of isolates was prepared with Gene JET kit (Fermentas) according to the manufacturer's instruction. Briefly, two solutions of 2ME+ lysis buffer (20µl+980µl for each isolate) and proteinase K + TE buffer (10µl+590µl) were prepared. RNA was extracted from isolates that have grown in last dilution of wells. For each test 6×10^8 cells was used. Isolated RNA was preserved in -20°C.

cDNA Synthesis

Synthesis of cDNA was conducted with the 1621K kit (Fermentase) and according to manufacturer's instruction. Briefly, 2µg of RNA was added to 1µl of Random hexamer primer and the final volume was reached to 12µl using DEPC treated water. The microtubes were placed in 65°C for 5 min and next on ice. For each tube, 4µl of 10X buffer, 2µl of dNTP (10mmol), 1µl ribonuclease and 1µl of Reverse Transcriptase M-MuL were added. The thermal profile of reaction included of 25°C (5min), 42°C (60min), 70°C (10 min for the reaction stop). The cDNA was preserved in -20°C until used in PCR reaction.

Real Time PCR Test

The RT-PCR reaction was set up for *ERG3*, *ERG6* and *ERG11* genes (Tables1 & 2). Primer designing for RT-PCR test was performed using GenBank (the international bank in NCBI). Master Mix was prepared the same for each reaction (*erg3*, *erg6*, *erg11* and β-actin) as shown in Table. The thermal reaction profile included of 95°C (5min), 95°C (50s), 60°C (45s), 72°C (50s) and final extension of 72°C (10min). The agarose gel of 1.8% and ethidium bromide were used for the visualization of products.

Table 1 The master mix prepared for PCR reactions (*erg3, erg6, erg11* and *β*-actin)

Master Mix	Total=20ul
Syber Green	10ul
Primer sense	0.8ul
Primer Antisense	0.8ul
cDNA	2ul
H_2O	6.4ul

Table 2 The sequence and conditions of primers used in this study

Name	nt	OD	GC%	Tm	Primers sequences (5' to 3')
ERG3-S	20	12	50	58.65	AGTGGGTGCAGTGATACAGT
ERG3-AS	20	12	50	59.22	TGCGGGTAAGAAGGTTGGTT
ERG6-S	20	12	55	59.82	AGCTACCGTTCATGCTCCAG
ERG6-AS	20	12	55	59.75	GTTCGGCAACTTCACGACTG
ERG11-S	20	12	45	59.88	CAGAAAAGTGGCGTTGTTGA
ERG11-AS	20	12	45	59.69	GCAGCATCACGTTTCCAATA

Results

MIC tests for fluconazole and aspirin

All the isolates were resistant to fluconazole and 64µg/ml aspirin serial mico-dilutions. The concentration of the aspirin at the next stage was increased to 500ug/ml and the next MIC and MBC were 15.62µg/ml and 31.25µg/ml, respectively by culture of 100ul isolates from suspension on the agar plate.

The RT-PCR reaction for *Erg3*, *Erg6* and *Erg11* Genes

In the RT-PCR reaction, the *erg6* was expressed (CT cycle of 22) with the housekeeping gene of β-actin which was amplified at the cycle of 22, and the expression was significantly increased when using aspirin at the concentration 15.62 µg/ml for the growth inhibition of *C. glabrata*. However, the expression profile of *erg3* and *erg11* was not detected before the cycle of 38, and thus no effect was induced by aspirin on the both genes. The amplification of *erg6* was detected at 15.62 µg/ml and *C. glabrata* were capable of growth at this concentration (MIC=15.62). However, aspirin conferred a fatal effect at the concentration of 31.25µg/ml, as no growth of the strains was observed.

Discussion

C. glabrata belongs to the normal microbiota of the oral cavity to gastrointestinal and vaginal tracts, and related clinical manifestations are more recurrent and problematic to treat mainly due to decreased azole susceptibility; and especially when any level of immunosuppressive agents are given to patients.[7] Aspirin as an NSAID drug was adopted for this study because before surveys have shown evidence of effect of these drugs on the expression of *erg* genes.[15,16] The *erg* genes are responsible for the azole resistance and thus the three *erg3, erg6* and *erg11* were assessed here in order to combat drug resistance due to *C. glabrata* isolates. In the present study, MIC for fluconazole and aspirin was conducted and all the isolates were resistant to the 46µg/ml of these drugs. But at concentrations of 500ug/ml, the MIC and MFC were 15.62 and 31.25µg/ml, respectively. Non-steroidal anti-inflammatory drugs according to previous studies have anti-fungal activity. In a survey by Sharma, All the isolates of *C. albicans* and nearly all of *C. parapsilosis,* 37.7% of *C. tropicalis* isolates exhibited significant reduction in MIC after concomitant culture with fluconazole and ibuprofen and no inhibition of growth was demonstrated among any isolate of *C. glabrata*.[17] Testing of Diclofenac sodium, Ibuprofen and Ketoprofen, by Ashraf et al., *Sodium Diclofenac* showed lower MIC against *C. albicans* and *C. glabrata,* while ibuprofen demonstrated lower MIC against *C. krusei* compared to other NSAIDs. On the other hand, these drugs showed antifungal and antiadherent activities, and were capable of detaching mature biofilms. Also, morphological switch of *C. albicans* was inhibited at a concentration of 500 µg/ml *Sodium Diclofenac*.[18]

In the RT-PCR test, the *erg6* gene was amplified before the cycle of 38, but *erg3* and *erg 11* were not amplified compared to the control β-actin gene. However, the expression profile of *erg3* and *erg11* was not detected before the cycle of 38. The amplification of *erg6* was detected at 15.62ug/ml Aspirin and *C. glabrata* were capable of growth at this concentration (MIC=15.62). However, aspirin conferred a fatal effect at the concentration of 31.25ug/ml, as no expression of *erg6* was detected and also no growth of the strains was observed. Different azole resistant genes and several homologues have been recognized to date.[19] Sequencing of CgERG4, CgERG11, CgERG5 and CgERG6 genes by Vandeputt determined exclusively a single missense mutation in the CgERG6 culminating in the substitution of cysteine

by phenylalanine aminoacide.[20] Moreover, a nonsense mutation in this gene has led to reduced susceptibility to azole compounds.[21] There may be a relationship between the mutations and the overexpression of *erg6* we revealed. However, in the study by Tobudic alterations in the *ERG 11* gene was considered as an important way of azole resistance.[22] The present study exhibited that aspirin induce the anti-fungal effect of fluconazole at higher concentrations conferring a synergistic effect; especially via alteration of *erg6* expression and leading to the *C. glabrata* strains susceptible to azole compound. However, we observed that aspirin has no effect on the expression of *erg3* and *erg11* genes. This was the first report of aspirin effect on the expression of *erg* genes.

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Conflict of interest

None.

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