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Synergistic Antifungal Effect of aspirin Combined with Fluconazole against azole-resistant oral *Candida glabrata* isolates

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ABSTRACT

Azole compounds have been a treatment option for *Candida glabrata* infections. However, azole resistance can occur through different mechanisms such as alterations in *ERG11* (lanosterol 14α-demethylase). Aspirin (ASA), a non-steroidal anti-inflammatory drug, showed antimicrobial activity against Candida. The purpose of this study was to evaluate the synergistic effect of ASA as an anti-inflammatory drug with the fluconazole on the azole-resistant *C. glabrata* isolates. In the cross-sectional study, a total of 60 oral samples were collected from the school of dentistry, Tehran University of medical sciences. After confirmation of fungal isolates, template DNA was extracted and PCR for detection of *Erg11* gene by specific primers was performed in all Fluconazole-resistant isolates. MIC for ASA was determined using broth dilution method in 96□well plates. RNA was extracted and cDNA synthesized according to the Omniscript RT kit instructions. The effect of ASA in the treated and non-treated groups on *ERG11* gene expression was determined by Real-Time PCR technique. Out of 60 collected samples, 12 (20%) *C. glabrata* were isolated. All of these isolates were resistant to fluconazole and carried *ERG11* genes. Real-time PCR results showed that the combination of ASA with fluconazole reduced *ERG11* gene expression. It is concluded that, treatment of candidal infections with ASA significantly reduced resistance in to azole compounds by down expression of *ERG11* gene, suggesting that an NSAID might be useful for azole-resistance candidal infections.

1. Introduction

Candidiasis is one of the most common opportunistic yeast infections that has increased among patients in recent years and has been recognized as one of the leading causes of death especially in people with immune deficiencies such as patients with AIDS (Vila et al., 2020). The frequency and severity of these infections have increased dramatically in the last two decades, following the widespread use of antibiotics, steroids and other immunosuppressive drugs (Kullberg et al., 2015). Candida species are found everywhere in human and animal symbiosis. *Candida glabrata* (*C. glabrata*) is one of the most common members of the genus *Candida* because of its ability to obtain drug resistance, its rapid and accurate identification is essential for the treatment of infections (Hitchcock et al., 1993).

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The azole antifungal compounds that target lanosterol 14-α-demethylase, encoded by the *ERG11* gene, are widely used to treat a variety of infections caused by this organism. Various mechanisms have been identified in the emergence of resistance to azole compounds in *C. glabrata* such as cell impermeability, alteration in ergosterol biosynthetic pathway enzymes, alteration in target enzymes (point mutation, overexpression and gene modification) and drug efflux pump. A point mutation in the *ERG11* gene cause misfolding of this protein and consequently decrease drug affinity. This gene encodes the cytochrome P450 lanosterol 14α-demethylase target enzyme (Rodero, 2003, Ribeiro, 2007).

Aspirin, also known as acetylsalicylic acid (ASA), inhibits pain, fever, and inflammation by inhibiting cyclooxygenases (COX-1 and COX-2) (Lewis et al., 1993). This nonsteroidal anti-inflammatory drug (NSAID) also has antifungal activity. ASA has potent antibiofilm activity against fungal biofilms and can be useful in combination therapy with antifungal agents in the management of some Candida-related biofilm infections. For example, ASA increases the antifungal effect of fluconazole at higher concentrations and provides a synergistic effect (Stepanović et al., 2004). Thus, the effect of ASA on the *ERG11* expression could be a new therapeutic option for azole-resistant *C. glabrata* infections. The aim of this study was to molecular identification of *ERG11* gene from fluconazole-resistant *C. glabrata* isolate and the effect of ASA on its expression by Real Time PCR method.

2. Materials and Methods

2.1. Fungal isolation

In this cross-sectional study, a total of 60 *C. glabrata* isolates were collected from the patients with oral candidiasis in a period of 8 months from February till September 2019. All strains were primarily identified by the morphological and microscopic features including small yeasts with budding cells without pseudohyphae/hyphae on corn meal agar (Difco, United States) with 1% Tween 80 and its pink colonies on the CHROMagar^{TM} Candida (CHROMagar, Paris, France). Germ Tube (GT) and Chlamydospore formation test were also performed. Then, each isolate was confirmed by PCR using ITS1/ITS4 primers (Kiasat et al, 2019). All strains were preserved in Sabouro dextrose agar (Merck Co., Germany) at 4 °C for further use. *C. glabrata* ATCC 5297 was used as a positive control in this work.

2.2. Antimicrobial susceptibility testing

In line with clinic and laboratory standard institute (CLSI), Fluconazole susceptibility testing was performed for all *C. glabrata* isolates on the Sabouro dextrose agar (Merck Co., Germany) by disk agar diffusion method.

2.3. Polymerase chain reaction

ERG11 gene was detected in the Fluconazole-resistant *C. glabrata* by PCR after extraction of genomic DNA using SinaClon DNA extraction kit (CinnaGen, Iran) according to the manufacturer's instructions. Primers used to identify *ERG11* gene included F; 5′-TGGAGACGTGATGCTG-3′ and R; 5′-AGTATGTTGACCACCCATAA-3′ (Lee et al,. 2004). The PCR reaction was fulfilled in a volume of 25 µl, including the following contents: 0.8 µl of template DNA, 12 µl PCR Master Mix (2×) (Thermo, Waltham, Massachusetts, United States), 0.7 µl of each primer, and 9.4 10.8 µl of distilled water. The samples were amplified in a thermo cycler (Eppendorf Master cycler) as follows: one cycle represents initial denaturation at 95°C for 7 min followed by 30 cycles of denaturation (95°C for 30 s), annealing (55°C for 60s) extension (72°C for 60s) and finally the process ends with a final extension at 72°C for 10 min. PCR amplicons were loaded on 1.0% agarose gel, stained with Gel Red™ (Biotium, Landing Pkwy, Fremont, CA, USA) and photographed with UV transilluminator.

2.4. Determination of ASA minimum inhibitory concentration (MIC)

In this study, ASA with the purity of 99.0% was purchased from Daroupakhsh Company, Tehran, Iran. Stock solutions of aspirin (900 mg/ml) were freshly prepared in dimethylsulfoxide (DMSO; BDH, UK). MIC for ASA was determined using twofold broth dilution method in 96□well plates (Cellstar®, Greinerbio□One, Germany) described by Al-

Bakri et al, (Al□Bakri et al,. 2009). In brief, the first experimental well was filled with double□strength Sabouro dextrose broth (SDB-Merck Co., Germany) while the other wells were filled with single□strength SDB (150 µl). A volume of 150 µl of ASA or DMSO was added. Double□fold serial dilution was then performed across the plate. Overnight batch culture of the fungal isolates (10 µl) was used to inoculate the wells to achieve a viable count of 1×10^6 CFU/ml. The plates were incubated for 24 h at 37°C. Stock solutions of 50 mg/ml of ASA was used. MIC was expressed as the mean concentration between the well showing growth and that showing no growth. Growth was detected as turbidity (630 nm) relative to an un-inoculated well using a microtitre plate reader (Bio□Tek, USA). Negative controls were performed with only sterile broth in each well, and positive controls were performed with only overnight culture in the wells. Positive control included DMSO in the same concentration (v/v) as that used in the experimental substances. Each MIC determination was performed in triplicate. Sub-MIC concentration of ASA was used for determine the expression of ERG11 genes.

2.5. Quantitative real-time polymerase chain reaction

For this purpose, 0.1 mg/ml of ASA was added into a 20 ml of DMSO solvent and then C. glabrata was inoculated to it. Messenger RNA (mRNA) was extracted from the fungal isolates which are at the end of the logarithmic phase of growth using Qiagen RNeasy micro kit (Qiagen, Valencia CA). RNase-free DNase (Qiagen) kit was used to avoid DNA contamination in the isolated RNA. The concentrations of RNA in each sample was quantified with a spectrophotometer at 260 and 280 nm wavelengths. Complementary DNA (cDNA) synthesis was performed with an Omniscript RT kit (Qiagen, USA) according to the manufacturer's instructions. The cDNA was stored at –20 ˚C until use. For normalization, expression of β-actin was examined with the primer pair of: F; 5□ -TTC TAC AAT GAG CTG CGT GTG G -3□ and R; 5□ -GTG TTG AAG GTC TCA AAC ATG AT-3□ . Reverse-transcriptase PCR (RT-PCR) was done using a 2× GreenStar Master Mix Kit (Bioneer, Korea) on a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). A typical RT-PCR sample (25 µl) contained 10.5 µl of PCR Master Mix, 0.8 µl of cDNA, 0.6 µl of 0.8 µM solutions of both forward and reverse gene-specific primers and 11.3 µl of deionized double distilled water. Real-time run protocol was the same as previously described Lari et al (Lari et al., 2018). All samples were run in triplicate. A critical threshold cycle (CT) value was used to represent ERG11 transcripts quantitatively. The ΔCT for ERG11 transcripts was calculated against that for the β-actin gene, and the ΔΔCT was calculated against that for the fluconazole-resistant *C. glabrata*. The *ERG11* relative expression was calculated by the Livak method (Livak et al., 2001).

2.6. Statically analysis

SPSS version 23 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Descriptive statistics, confidence interval and Pearson's chi□square tests were employed in this study. Statistical significance was defined as P-value less than 0.05.

3. Results

Of the 60 periodontal specimens under study, 12 (20%) were identified as *C. glabrata*. These isolates were negative for hyphae production, and positive for blastopore production. Pink colonies were observed on CHROMagarTM Candida. Also, all doubtful colonies were confirmed using PCR test. All *C. glabrata* isolates were resistance to fluconazole and had *ERG11* gene. ASA had an effect on gene expression (figure 1). In this study, the fold change in the treated group was equal to -1.13 in compare to the untreated group, indicating a decrease in the relative expression and inhibitory effect of ASA and also, a -1.13 % decrease in the expression of the gene affected by ASA treatment (table 1).

4. Discussion

C. glabrata is responsible for 25 - 30% of fungal infections and have worldwide

distribution. The lanosterol 14α-demethylase gene (*ERG11*) gene is vital for production of the fungal sterol ergosterol, a critical component of the fungal plasma membrane. Loss of *ERG11* is a lethal event or causes a profound growth defect in most Candida species. *ERG11* gene in *C. glabrata* with different molecular mechanisms cause resistance of the organism to antifungal drugs such as azole compounds. Resistance to azoles can occur through different mechanisms, such as mutations in the *ERG11* gene (Lanosterol 14α-demethylase (CYP51).
Several studies have shown that a combination of ASA with fluconazole induces synergistic activity against Candida yeast (Liu et al,. 2014, Zhou et al, 2012).

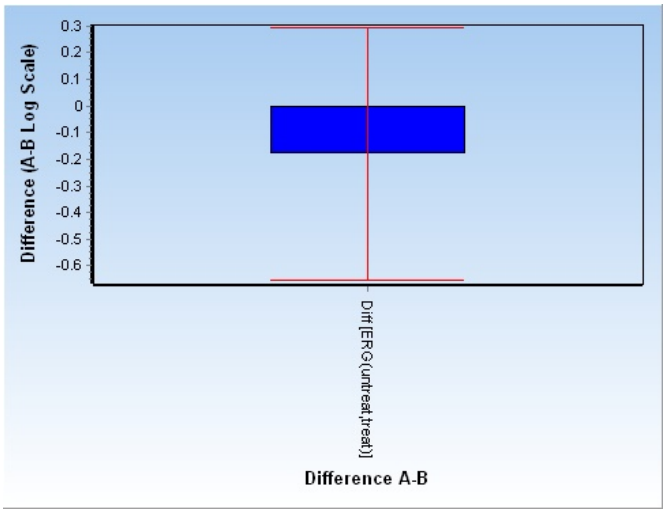

Figure1. Fold change differences in treated and untreated samples

Table 1. Statistical analysis

	ERG (untreated)	ERG (treat)	Diff ERG (untreated, treat)
Sample1	3.575	3.715	-0.14
Sample 2	4.885	5.1	-0.215
count	2	2	2
Mean	4.23	4.40	-0.177
STDEV	0.92	0.97	0.05
P-Value			0.132
Confidence Level(CI)			95%
Difference (A-B log scale)			-0.177
Fold Change			-1.13

In a study directed by Goodarzi et al, showed that *Erg6* gene amplified at 1 µg / ml ASA compared to β-actin control gene (Goodarzi et al., 2016). Therefore, ASA at this concentration is able to increase the response of the *Erg6* gene and fluconazole resistance, which is consistent with the present study. Aslani et al., in a study investigating the effect of *Satureja bachtiarica Bunge* and *Echinophora platyloba* on the expression of *MDR1* and *ERG11* genes in fluconazole-resistant Candida, concluded that *Echinophora platyloba* alcoholic extracts significantly reduces *ERG11* gene ($p < 0.05$). The researchers concluded that the herb can be described as an effective antifungal compound with low side effects (Aslani et al., 2014). In a study conducted by Teymuri et al, of 142 candida, 5 isolates were resistance to

fluconazole. In line with our study, increased *ERG11* gene expression was identified as one of the important mechanisms of fluconazole resistance (Teymuri et al., 2015). According to the results of the present study, Balabandi et al, Showed that different mechanisms simultaneously contribute to azole resistance in Candida strains (Balabandi et al,. 2017) .The presence of mutations in the *ERG11* gene and altered expression of other genes may increase the resistance to high concentration of fluconazole in this study and ASA combination can significantly decrease the expression of *ERG11* gene and increase the efficiency of fluconazole. Pina-Vaz et al, showed that the practicability of using ibuprofen, alone or in combination with azoles, in the treatment of candidiasis, especially when applied topically, taking advantage of the drug's antifungal and anti-inflammatory properties (Pina-Vaz et al et al,. 2000). In line with our data, Liu et al, indicates that the combination of fluconazole and licofelone has synergistic effect against resistant *C. albicans* and could be a promising therapeutic strategy for the antifungal treatment. Alem et al, showed that aspirin possesses potent antibiofilm activity in vitro and could be useful in combined therapy with conventional antifungal agents in the management of some biofilm-associated Candida infections (Alem et al., 2004).

Conclusion

It is concluded that, treatment of candidal infections with ASA significantly reduced resistance in to azole compounds by down expression of *ERG11* gene, suggesting that an NSAID might be useful for azole-resistance candidal infections. In fact, combined ASA treatment with conventional antifungal drugs has shown acceptable therapeutic effects against Candida species, which are dependent on ASA concentration. However, further studies in this field are necessary until a correct choice is made.

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Conflict of interest

All authors declare that there is no conflict of interest in this study.

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