Cetylpyridinium Chloride Induces Resistance Genes in *Candida Albicans*

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Abstract

Aim: The antimicrobial cetylpyridinium chloride (CPC) is used in the management of denture stomatitis-associated oral candidiasis as an alternative therapy as well as for oral hygiene. Cetylpyridinium chloride survives in the oral cavity for long periods at low doses, which fluctuates due to the dynamics of the oral cavity. In this study, it was aimed to evaluate the impact of different sub-therapeutic CPC concentrations (1/100, 1/200) for different time periods (0.,5., 2., 24., 48. h) on the expression of drug-resistance genes (*CDR1, CDR2, MDR1*, *ERG11*) in *Candida albicans* SC5314.

Method: Total RNA was extracted immediately after antimicrobial exposure using the Biospeedy® Tri-Easy Isolation Kit followed by Real-Time Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR). The data were analyzed by the comparative $2^{-\Delta\Delta CT}$ method to calculate the relative expression of the target genes after treatment with different CPC concentrations, standardized to the housekeeping gene Actin.

Results: In this study, it was found that the drug resistance gene expression levels increased after exposure to high CPC concentrations (1/100) for 48 h, whereas the gene expression levels were downregulated at 1/200.

Conclusion: These results may provide an insight into the mechanisms of action of drug-resistance genes in *Candida albicans* and aid the development of future strategies for using CPC as an alternative therapy.

Keywords: Cetylpyridinium chloride, drug-resistance genes, oral candidiasis, *candida albicans*, antimicrobial agents.

Setilpridinyum Klorür *Candida Albicans* Direnç Genlerini İndükler

Öz

Amaç: Antimikrobiyal setilpridinyum klorür (CPC), ağız hijyeninin yanı sıra alternatif bir tedavi olarak protez stomatitiyle ilişkili oral kandidiyazis tedavisinde kullanılır. Setilpridinyum klorür (CPC), ağız boşluğunun dinamikleri nedeniyle dilue olarak ağız boşluğunda düşük dozlarda uzun süre mevcut kalır. Bu çalışmada, farklı zaman periyotlarında (0.,5., 2., 24., 48. saat) farklı alt terapötik CPC konsantrasyonlarının (1/100, 1/200) *Candida albicans* SC5314'te ilaca direnç genlerinin (*CDR1, CDR2, MDR1, ERG11*) ekspresyonu üzerindeki etkisinin değerlendirilmesi amaçlanmıştır.

Özgün Araştırma Makalesi (Original Research Article)
Geliş / Received: 09.11.2021 & **Kabul / Accepted:** 28.02.2023
DOI: https://doi.org/10.38079/igusabder.1021179
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Yöntem: Toplam RNA, antimikrobiyal ile maruziyetten hemen sonra Biospeedy® Tri-Easy Isolation Kit ve ardından qRT-PCR kullanılarak ekstre edildi. Bu veriler, farklı CPC konsantrasyonları ile muameleden sonra hedef genlerin nispi ekspresyonunu hesaplamak için karşılaştırmalı 2-ΔΔCT yöntemiyle analiz edildi, Aktin housekeeping genine standardize edildi.

Bulgular: Bu çalışmada, 48 saat boyunca, yüksek CPC konsantrasyonuna (1/100) maruz kalan örneklerde *Candida* ilaç direnci gen ekspresyon seviyelerinin arttığı, buna karşın 1/200 konsantrasyonunda bekletildiğinde azaldığı bulundu.

Sonuç: Bu sonuçlar, *Candida albicans*'ta ilaca dirençli genlerin etki mekanizmaları hakkında bir fikir verebilir ve alternatif bir tedavi olarak CPC'yi kullanmak için gelecekteki stratejilerin geliştirilmesine yardımcı olabilir.

Anahtar Sözcükler: Setilpridinyum klorür, ilaç direnç genleri, oral kandidiyazis, *candida albicans*, antimikrobiyal ajanlar.

Introduction

Oral mouthwashes (MoWs) are used as an additional treatment for oral candidiasis associated with prosthetic stomatitis because of its drug resistance. Cetylpyridinium chloride (CPC), a component of the MoWs, is also used to maintain oral hygiene[1,2]. CPC has a broad spectrum of antimicrobial activity, in particular fungicidal activity against yeast. Oral rinsing with 0.05% CPC results in an immediate germ reduction (> 99%)[3,4]. As a surfactant, CPC non-specifically binds to the charged bacterial protein and changes the surface tension of the bacterial cell wall, resulting in cell wall leakage and affecting cell metabolism[5-7].

Recent studies have emphasized on the occurrence of CPC resistance[7]. However, its mechanism has not been clarified yet. Researchers have studied several factors that may effectively aid the formation of resistance in different model systems[8-10]. *Candida* resistance mechanisms against antifungals have been identified and shown to be transferred through transmission of genetic material, specifically resistance genes, which also function against many antimicrobial agents[11].

However, pathogenic fungi have received little attention as biocide targets[12]. Although previous studies have noted the CPC effect against *Candida albicans* (*C. albicans*)[13] as well as suggested its therapeutic potential against candidiasis, there are only a few reports on the development of CPC resistance in yeast[7]. Candidiasis is typically treated with fluconazole or related azole antifungals that function as ergosterol biosynthesis inhibitors[14]. However, extended therapy often results in fluconazole-resistant strains that exhibit upregulated expression of multiple drug resistance genes, especially *CDR1* and *CDR2* (encode multidrug efflux transporters of the ATP binding cassette transporter family), *MDR1* (encodes leading facilitator transporter), and *ERG11* (encodes sterol 14 α-demethylase to the drug target enzyme) as well as their mutations[14-16]. Additionally, mutants that do not contain *CDR1* and *MDR1* lose their azole resistance along with resistance to antifungals and antiseptics[17]. Several studies have focused on the molecular mechanisms involved

in resistance development conferred by the resistant genes *CDR1, CDR2, ERG11,* and *MDR1*. However, variables involved in these studies on different models lead to contradictory results.

The dynamics of the mouth and the effect of saliva reduce the therapeutic CPC doses to sub-therapeutic doses. Considering their use during the day, the therapeutic concentration is diluted by saliva and remains in the mouth for a long period of time[18,19]. Therefore, based on these reports, it was hypothesized that the presence of these features in the mouth for a long time might not affect the resistance genes in planktonic cells. In this study, it was aimed to investigate the effectiveness of CPC sub-therapeutic concentrations on *C. albicans* resistance gene in a planktonic model. Furthermore, using the low-dimensional model that established, it was aimed to determine the effect of multi-dimensional factors that cause resistance development.

Material and Methods

Strain

C. albicans SC5314 (obtained from the stock collection of the lab) stored in yeast extract peptone dextrose (YEPD) broth containing 10% glycerol at -80 °C was used in the present study.

Preparation of the Yeast Suspension

C. albicans SC5314 was cultured in YEPD broth (10 g yeast extract, 20 g peptone, and 20 g/L dextrose) at 30 °C for 24 h. Thereafter, the cultured cells were inoculated in 200 mL YEPD at a concentration of 2×10^4 cells/mL. The cells were further cultured with agitation overnight at 30 °C; the initial optical density at 600 nm (OD_{600}) of 0.1 increased to log phase OD_{600} of 0.8–1 (approximately 1×10^7 cells/mL).

Antiseptic Agent

Therapeutic concentration of CPC (0.05%) was prepared. Sub-therapeutic concentrations (S-TCs) of the antiseptic agent, 1/100 and 1/200, were prepared by adding 10 µL of the therapeutic concentration to 0.99 and 1.99 mL of phosphate-buffered saline (PBS), respectively[18]. The therapeutic dose of CPC (0.05%) was not used in this study as it is lethal.

Exposure of C. albicans SC5314 to the Antiseptic Agent

Tubes of *C. albicans* SC5314 cell suspension (10^7 cells/mL) were prepared in 1 mL PBS. After that, the tubes were centrifuged at 3000 × g for 10 min, the supernatant was decanted, and yeast pellets were resuspended in either 1 mL sterile PBS (positive control) or 1 mL PBS/CPC (1/100 and 1/200). The cells were exposed to the two S-TCs (1/200 and 1/100) for different time periods (0, 0.5, 2, 24, and 48 h) at 37 °C on a shaker. After incubation for the appropriate time period, the cells were centrifuged at 3000 x g for 10 min at 4 °C and resuspended in sterile PBS to remove the antiseptic. Two such washes were performed. Following antiseptic removal, the supernatant was again decanted and the pellets were washed twice with PBS to eliminate potential carry-over

effects the antiseptic, and finally resuspended in 1 mL sterile PBS.[20] These pellets were stored at -20 °C until RNA extraction procedure.

RNA Extraction

Total RNA extraction from *C. albicans* SC5314 strain was performed using a commercially available kit Bio-Speedy® RNA-TRiRegular (Bioeksen, Turkey).

Synthesis of cDNA

First-strand cDNA synthesis was carried out using the Biospeedy® cDNA synthesis kit according to the manufacturer's instructions (Bioeksen, Turkey). Briefly, 300 ng total RNA was incubated with 6 µL RNase-free water and 2 µL oligo dT at 70 °C for 10 mins. The reaction mix included 4 µL 5X speedy reaction mix, 1 µL dNTP mix, 1 µL reverse transcriptase, 14 µL RNAse-free water, and 8 µL of the prepared RNA mix. The final cDNA synthesis was performed at 37 °C for 60 min.

Real-Time PCR

Primer and Amplicon Design

For the specific and efficient amplification of the PCR products, it is very crucial to determine the target sequence and to design the primers specific to these sequences. Sequences of the target genes were obtained from the NCBI DNA database (https://www.ncbi.nlm.nih.gov), and specific primers to target genes of the study were designed using Primer-Blast, as listed in Table 1.

Table 1. The primer sets and their targets for resistance genes of *Candida albicans*

Name	Primer	Oligonucleotide sequence (5' to 3')	Position	Product (bp)	Tm (°C)
ACT1	Forward	TGCTGAACGTATGCAAAAGG	882-901	186	51
ACT1	Reverse	TGAACAATGGATGGACCAGA	1048-1067		
CDR1	Forward	CATGGTCAAGCCATTTTGTG	3148-3167	200	51
CDR1	Reverse	ATCCATTCTGCTGGATTTGC	3328-3347		
CDR2	Forward	GAGAAAGTTAGCTGATCAT	3142-3161	199	51
CDR2	Reverse	TGCTTCCTTAGGACATGGA	3322-3341		
ERG11	Forward	TACTGCTGCTGCCAAAGCTA	1284-1303	278	53
ERG11	Reverse	CCCAAATGATTTCTGCTGGT	1542-1561		
MDR1	Forward	CAAATTCCCACTGCTTTGGT	592-611	148	51
MDR1	Reverse	CGGCTAACCCAACTGGTAAA	720-739		

In silico analysis of the primers Primer3 (v.0.4.0) software was used and those containing very few internal structures (i.e., hairpins, primer–dimer formation) were chosen for qPCR analysis.

Real-Time PCR Analyses

Roche LightCycler 480 Real-Time PCR Detection System (Roche, Germany) was used to determine the resistance of gene expression profiles at the mRNA level. Every 20-µL reaction included 4 µL reverse-transcribed cDNA, 10 µL EvaGreen qPCR pre-Mix (Bioeksen, Turkey), 500 nM of each primer, and nuclease-free water. The Bio-Speedy® EvaGreen qPCR preMix consisted of Eva Green Dye® (fluorescent dye), one-fusion DNA polymerase, dNTP blend with dUTP for preventing false positives, and optimized buffer components. The use of Eva Green dye, as compared to the widely used SYBR Green dye, was expected to reduce the non-specific binding. The experimental conditions were as follows: the starting denaturation temperature was 95 °C for 10 min, followed by 40 amplification cycles of 15 s at 95 °C, 1 min at 49–50 °C (according to the primer annealing temp.), and 30 s at 72 °C. The efficiency of each primer and the specificity of the amplicon were examined by serial dilution of cDNA and melting curve analysis, respectively.

For melting curve analysis, the products were heated to 95°C for 1 min, then cooled down to 65°C, and slowly heated again to 95 °C during fluorescence monitoring. To ensure the reliability of the experiments, negative controls, no-template control (NTC), and no-reverse transcriptase (NRTC) control were routinely used to identify potential sources of contamination and to avoid amplification of genomic DNA contaminants during each assay. For all negative controls, 40 qPCR cycles consistently yielded no critical threshold (Ct) values (data not shown). Relative mRNA levels were analyzed in separate wells by comparative Ct ($2^{-\Delta\Delta CT}$) method, as defined by Livak & Schmittgen[21]. Amplification of treated and untreated controls was performed in separate wells. The relative quantifications were performed by comparing the expression of the target genes to that of the housekeeping gene Actin (ACT1); mRNA levels of the target drug resistance genes were normalized to this positive control gene. Experiments were carried out in triplicates and results were expressed as their mean value.

Statistical Analyses

Bivariate correlation analyses were performed using the software MINITAB 17 (Minitab Ltd., England). Correlations were evaluated using Pearson's method. Statistical significance was considered at $p<0.05$.

Results

The expression levels of the *C. albicans* resistance genes (*ERG11, CDR1, CDR2,* and *MDR1*) were measured at 0., 0.5., 2., 24., and 48. h after exposure to different S-TCs, 1/200 and 1/100, by qRT-PCR. Relative quantification of the target genes expression was calculated after the normalization with ACT1. The amplification efficiency of each target gene was 1.01 ± 0.05. Results were expressed as nFold ($2^{-\Delta\Delta CT}$); values >1 and <1 indicated the overexpression and the

underexpression of target genes after antiseptic exposure for different incubation periods, respectively.

C. albicans Resistance Gene Expression

After 30 min and 24 h exposure to CPC, increase in CDR1 expression was observed at both S TCs ($r>0.6$, $p<0.05$) (Fig. 1, 2) (Table 2).

Figure 1. Expression profiles of genes encoding resistance factors in *C. albicans* incubated in 0, 1/200 (10) and 1/100 (20) concentrations for 0.5 h of cetylpyridinium chlorid.

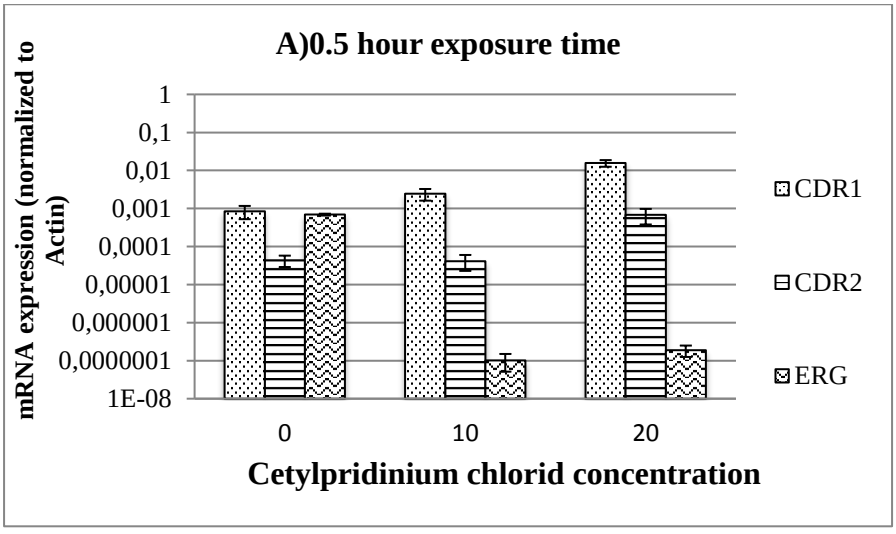
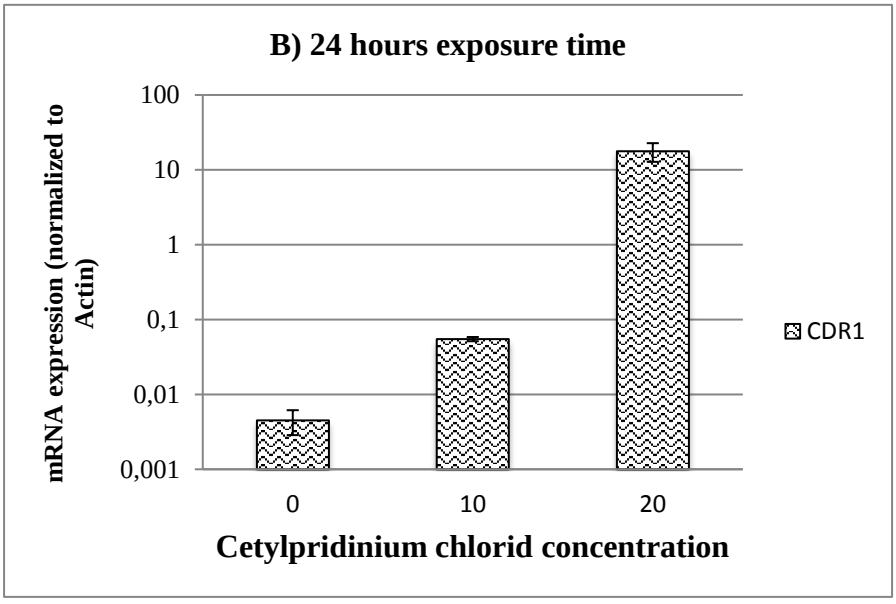


Figure 2. Expression profiles of genes encoding resistance factors in *C. albicans* incubated in 0, 1/200 (10) and 1/100 (20) concentrations for 24 h of cetylpyridinium chlorid.



After 48 h exposure to 1/200 concentration of CPC, CDR1 was downregulated; however, at 1/100 concentration, it was upregulated (overexpressed) ($r>0.6$, $p<0.05$) (Fig. 3) (Table 2).

Figure 3. Expression profiles of genes encoding resistance factors in *C. albicans* incubated in 0, 1/200 (10) and 1/100 (20) concentrations for 48 h of cetylpyridinium chlorid.

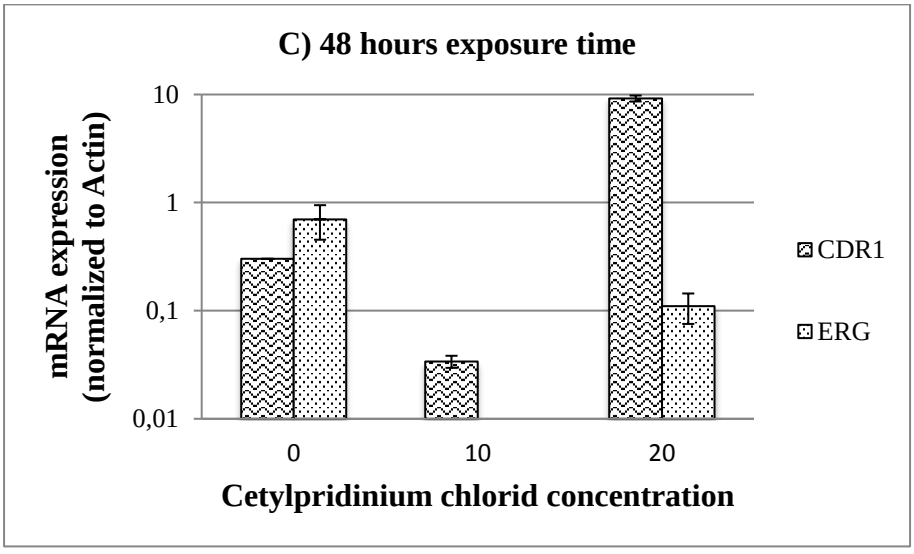


Table 2. *CDR1* expressions in *C. albicans*, exposed (0.5 h, 2 h, 24 h, 48 h) to S-TCs (1/200 and 1/100) of CPC

Times/Concentrations	O	1/200	1/100
0	0,0174642		
0.5 h	0,00084738	0,00243708	0,01563118
2 h	0,00548139	0,23001727	0,01379093
24 h	0,00450685	0,05490932	17,7529097
48 h	0,30175907	0,0339322	9,20099767

CDR2 was overexpressed after 2 h exposure to 1/200 concentration of CPC ($r>0.6$, $p<0.05$) (Table 3). After 2 and 24 h exposure to 1/200 concentration of CPC, increase in *ERG11* expression was observed ($r>0.8$, $p<0.01$) (Table 4).

Table 3. *CDR2* expressions in *C. albicans*, exposed (0.5 h, 2 h, 24 h, 48 h) to S-TCs (1/200 and 1/100) of CPC

Times/Concentrations	O	1/200	1/100
0	0,000232318		
0.5 h	4,33209E-05	4,14905E-05	0,000678133
2 h	0,00061874	11,70724804	0,140224452
24 h	0,002364844	0,003636342	1,39259155
48 h	0,002585244	0,00512421	0,815347351

Table 4. *ERG11* expressions in *C. albicans*, exposed (0.5 h, 2 h, 24 h, 48 h) to S-TCs (1/200 and 1/100) of CPC

Times/Concentrations	O	1/200	1/100
0	0,01418008		
0.5 h	0,00069122	1,006E-07	1,8732E-07
2 h	0,01122512	0,57552207	0,00018303
24 h	0,0072292	0,03276926	0,00628991
48 h	0,69998179	0	0,10982568

MDR1 showed significant increase in expression after 2 h exposure to 1/200 concentration of CPC ($r>0.8$, $p<0.01$) (Table 5). Also there was increased expression even at 0 concentration (positive control). Moreover, after 48 h at 0 concentration, *CDR1* and *ERG11* were upregulated (Table 2, 4).

Table 5. *MDR1* expressions in *C. albicans*, exposed (0.5 h, 2 h, 24 h, 48 h) to S-TCs (1/200 and 1/100) of CPC

Times/Concentrations	O	1/200	1/100
0	0,0154188		
0.5 h	0,00176186	0,00052679	0,00069834
2 h	0,13514292	1911,20173	0,34794249
24 h	0,07688067	3,16420291	9,91758747
48 h	0,0025505	1,59002428	0,84197826

Discussion

The present study used a simplified model to measure the activity of CPC on the expression of drug resistance genes in planktonic *C. albicans* expression in vitro. Results differ for different resistance genes in the planktonic model exposed to CPC. All genes in the study except *CDR1* were downregulated at lower concentrations after 0.5 hour of CPC exposure. In addition, *CDR1* was slightly overregulated than control.

Cetylpyridinium chloride reportedly spreads slowly in biofilms. In one study, it was found to accumulate in *Streptococcus mutans* biofilms and could not be practically removed[22]. CPC and negatively charged biofilm species-have been found responsible for this affinity[23,24]. Hence, in this study, it was aimed to investigate the effect of subtherapeutic concentrations of CPC on resistance genes in the *C. albicans* planktonic model.

Oral microorganism species, including *Candida*, are inhibited by CPC MoWs at remedial concentrations[3]. The broad-spectrum activity of the evaluated CPC rinses indicates severe inhibition of oral microorganisms. These effects are consistent with decrease in dental plaques reported previously[3,25]. Given that the resistant strains of *C. albicans* are becoming increasingly problematic, improving the therapeutic applications of CPC would be immensely beneficial. Considering the oral factors involved in clinical setting, in the present study, it was used sub-therapeutic doses (1/200 and 1/100) to evaluate the effects of CPC for extended periods (48 h) on drug resistance genes. It was supposed that these exposure times were critical for these genes. Such long term CPC exposure was valid, considering that MoWs may be used twice a day at therapeutic concentration, after morning and evening meals, during the treatment period. Therefore, the concentration of CPC between the two uses can be considered as S-TCs.

There is limited information about the effects of MoWs on oral microbiota composition in healthy individuals. Ardizzoni *et al.*[26] observed the effects of MoWs containing CPC on *C. albicans* isolates from swabs and demonstrated its efficacy in impairing the ability of *C. albicans* to adhere to both abiotic and biotic surfaces. They further reported that in mixed biofilm model, CPC did not impair its biofilm-forming capacity. Several studies in different models have shown that saliva promotes the attachment of *C. albicans* to prosthetic acrylic[8,9]. Directly attacking microorganisms with antimicrobials can cause resistance to stress and result in development of biofilm. Moreover, the critical role of persister cells in the survival of both biofilm and planktonic populations demonstrates a new paradigm in understanding biofilm infections[10]. The persister hypothesis provides a satisfactory explanation for the astounding resistance of bacterial biofilms to all known antibiotics[27]. The presence of persister cells in *C. albicans* was first introduced by LaFleur *et al.*[28] Many questions about *Candida* persister cells have not yet been answered; however, the results of the present study-on the resistance gene expression dynamics in single-cell planktonic model may contribute to understanding of how drug resistance mechanisms may develop differently in biofilms and planktonic cells, despite similar environmental growth conditions. Indeed,

planktonic cells exhibit different species-specific behavior, which can be extended to the mechanisms involved in biofilm resistance, and used to develop new therapeutic concepts[23]. Also, research on biofilm resistance may not be considered essential in the discovery of new drugs, as planktonic populations are much easier to manipulate[10,29]. Therefore, in the present study, it was considered CPC-specific factors as well as *C. albicans*-specific factors in this planktonic model.

Furthermore, *ERG11* upregulation in planktonic cells treated with high concentrations of antifungal causes antifungal drug resistance[14,30,31]. Edlind *et al.*[7] reported that the expression of *C. albicans CDR1* and *CDR2* involved in drug resistance increased at a higher minimal inhibition concentration (MIC). Moreover, induced expression of *CDR1, CDR2,* and *MDR1* have been reported to be greater in biofilm-associated *C. albicans* cells than in vitro and in vivo planktonic cells[32-34]. Increased expression of CDR genes was observed mainly after 24 h and to a lesser extent after 48 h, while *MDR1* was over-expressed only after 24 h[33]. These observations showed that the upregulation of drug flow pumps does not play an important role in drug resistance in mature biofilms because both groups observed a decrease in flow pump gene expression in aging biofilms, while resistance generally increases as the biofilm ages. The biofilm did not seem necessary to fight antifungals because surface adhesion was sufficient to trigger overexpression of this gene[32,35]. These findings contradict those of the above mentioned studies, which may be attributed to differences in model-dependent mechanisms as in vitro model systems differ for biofilms and planktonic cells[36].

In the present study, after 48 h at 1/200 concentration of CPC treatment, the expression of *CDR1* significantly decreased and *ERG11* were not expressed (Fig.3), while *CDR2* and *MDR1* were slightly upregulated. All these gene expression levels, except *CDR1*, decreased on critical point at lower concentration, at 0.5 h. On the other hand, an increase in their expression levels was observed at higher dose (1/100), after 0.5 h. However, the ergosterol percentage was significantly decreased upon CPC treatment (Fig.1,3). Based on this critical point, this finding suggest future studies using CPC that may play a role in alternative therapy for candidiasis with resistance to azoles.

In this study, it was found that treatment with low doses of CPC at 0.5 h reduced resistance gene expression. This finding is not consistent with other studies[32,33] that did not mention resistance genes in planktonic cells. Moreover, preventing biofilm formation may be an interesting therapeutic option that aims to increase the sensitivity of *C. albicans* to antifungals. The present study demonstrated the effect of long-term exposure to CPC at low concentration in the absence of the effects of saliva proteins, adhesion, biofilm, and persister cell development factors. Finally, it was concluded that the downregulation of resistance genes in a single strain of *C. albicans*, was affected by long-term treatment at low CPC concentration, in vitro.

Limitations

This study is attempted to emphasize this with a preferred model, although it is not a simulation of true in vivo conditions, owing to the limitations of conducting this study under laboratory conditions. Primarily, such microbiological ecosystem is more complicated.

Conclusion

The elucidation of these resistance mechanisms is promising in the development of optimal treatments[37]. With the emergence of new resistance profiles, alternative therapeutic approaches are needed[38]. Innovative therapeutic strategies are often required to avoid the difficulties of traditional infection therapy caused by drug-resistant organisms. Another innovative solution that increases the efficacy of treatment for microbial infections may be combination therapy, which involves the use of a lower dose of the antimicrobial at the same time with the antifungal for appropriate times[39-42]. Furthermore on-site and in vivo studies are required to investigate the exact mechanism.

The present study provides insight in to the mechanisms involved in the development of drug resistance by evaluating the expression of resistance genes after treatment with a common antimicrobial agent, CPC. Moreover, the research focuses on *C. albicans,* one of the most common infections in the medical field. Therefore, the relevance of the present study is seld-evident. The results of this study may provide potential alternatives to the problem of drug resistance faced by the medical community.

Funding: This work was supported by Marmara University, Scientific Research Projects Committee (No. SAG- B-040712 0261).

Disclosure Statement: No competing financial interests exist.

Ethical approval: Not required.

Acknowledgments: Thanks to Prof.Dr. Tanju Kadir for valuable discussions and feedback on the manuscript.

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