Synergistic effect of low doses of Chlorhexidine and Clotrimazole against Candida spp.

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Abstract

Objectives Systemic diseases or oral situation changes can result in oral infections like candidiasis. Mouthwash is the most prevalent method to prevent or cure these infections. To formulate a more effective mouthwash, we combined Clotrimazole with a low dose of Chlorhexidine to investigate the in vitro effect against Candida spp.

Methods and Materials Combinations of Chlorhexidine (0.03-16µg/ml) and Clotrimazole (0.03-16µg/ml) were tested against Candida spp. by microdilution chequerboard technique and disk diffusion method.

Results From the chequerboard combination assay, the MICs of Chlorhexidine and Clotrimazole against Candida spp. decreased from >16µg/ml to 2-1µg/ml and from 2-0.25µg/ml to 0.5-0.125µg/ml, respectively, demonstrating favorable synergistic effects against 21 (84%) strains of Candida spp. The disk diffusion method showed an increase in halo size for the combination group when compared to the Clotrimazole alone group.

Conclusions Studies have shown that combinations of antiseptic and antifungal agents are effective in nature. In our study, we found that low concentrations of Chlorhexidine can enhance the antifungal effect of Clotrimazole against Candida spp.. We predict that the mechanism of this synergism could be due to the increased penetration of Clotrimazole brought about by the binding of Chlorhexidine to the cell membrane. Further studies to determine the mechanism and in vivo effects could increase its probable usage in clinical studies.

Background

The oral microbiome is a dynamic ecosystem consisting of multiple species of microorganisms living in a commensalistic relationship with its host[1]. Sometimes, due to systemic diseases or changes in oral condition, the imbalance of the ecosystem could induce a pathologic condition[1]. Studies reveal that Enterococcus faecalis, Actinomyces, and Candida albicans are the most prevalent microorganisms associated with dental
infection[2]. Unlike bacteria, the opportunistic pathogen *C. albicans* is a versatile microbe capable of adapting itself to any environment[3]. It exhibits a variety of virulence factors like adherence, thigmotropism and phenotypic switching, and secreting a degenerative enzyme that degrades the dentinal collagen[4]. In cases where the host immunity system is deficient, *C. albicans* could cause further damages.

Chlorhexidine is an old antiseptic agent with a broad antibacterial spectrum, which acts against gram-positive and gram-negative bacteria, and some fungi[5]. 0.1%-1% chlorhexidine is the most commonly used formulation for mouthwashes [6]. It is widely used to prevent periodontal diseases, dental caries, and mucositis[7]. Furthermore, chlorhexidine is recommended as an antiseptic agent in oral surgery procedures and as a cleaning agent for dental prostheses[1].

Clotrimazole is a broad-spectrum antifungal drug mainly used for treating *Candida* and other fungal infections[8]. It is a very well-tolerated product with few side effects and is widely used as a topical treatment for tinea pedis, vulvovaginal and oropharyngeal candidiasis[8]. It exhibits fungistatic antifungal activity by targeting the biosynthesis of ergosterol, thereby inhibiting fungal growth[9].

In the present study, we combined low doses of chlorhexidine and Clotrimazole against different *Candida* species, to evaluate the synergistic effect of the antiseptic compound and antifungal agent. This *in vitro* study may help with new clinical mouth wash formulations for patients with different types of oral infections.

### Results

**Antifungal Combination test**

The interactions of Clotrimazole and Chlorhexidine against all strains were tested via the microdilution chequerboard technique, adapted from the CLSI M27-A4 microdilution method[11]. As described, 50 μl of Clotrimazole or 50μl of Chlorhexidine was serially diluted in horizontal or vertical direction on the 96-well plate, which contained 100 μl 1-5×10³/ml inoculum suspension. Interpretation of results was performed after incubation at 35°C for 24 h. Drug combination interaction was classified on the basis of the fractional inhibitory concentration index (FICI). The FICI is calculated by the formula: FICI = (MIC A combination/MIC A alone) + (MIC B combination/MIC B alone)[12]. All tests were performed in triplicate.
Disk Diffusion
The disk-diffusion assay was performed according to the CLSI M-44A2 with minor modifications[10]. Briefly, a sterile cotton swab was dipped in Candida spp. spores suspension (1-5x$10^6$ cells/mL) and inoculated on RPMI-1640 plus 2% glucose plates. The plates were dried for 5 minutes. Then, paper disks (6 mm) were placed onto the MHA surface and embedded with Clotrimazole (32µg/disk) or Chlorhexidine (5µg/disk) and the combination of two compounds. Plates were then incubated at 35°C, examined after 24h and the growth inhibition haloes around each disk were measured. The results were expressed as halo diameters in mm.

Statistical analysis
The data presented are expressed as mean value of 3 independent experiments. Analysis of variance (ANOVA) and Bonferroni post-hoc test were carried out by SPSS V.13 to assess values obtained between different groups. All tests were two-tailed and $P\leq0.05$ was considered statistically significant.

Discussion
Clotrimazole is a very commonly used topical antifungal agent that is available in plenty of formulations such as creams, pessaries, lotions, powders, lozenges, topical solutions and vaginal inserts/tablets[8]. Clotrimazole is also formulated with the steroid hydrocortisone. These products are aimed at the treatment of superficial fungal infections, as well as oropharyngeal candidiasis and vulvovaginal candidiasis. Although resistance to clotrimazole is now quite common in certain patient subpopulations with candidiasis, clotrimazole is still a wide-spread used antifungal drug in the general population[8]. From our study, the MIC range of clotrimazole against Candida spp. is 0.5-2 µg/ml, where high MIC values are seen in 2 strains of C. albicans (CA6 and CA7). It is still considered a very effective anti-Candida drug. Chlorhexidine [7] is one of the most effective antimicrobial and antiplaque agents. The mechanisms of action of chlorhexidine include binding to the cell membrane of bacteria, preventing bacterial adhesion and causing bacterial cellular contents leakage. High concentrations (1%-2%) of chlorhexidine have shown good inhibitory effects against Candida[5]. Lower concentrations (0.1%-0.5%) are less effective in inhibiting these opportunistic pathogens in the oral cavity. High concentrations of chlorhexidine were found to be irritants, therefore low concentrations which has better
patient compliance are used in pharmacological formulations [5].

The concept of the combination of antifungal with antiseptic agents has been discussed in several papers. The combined effect of nystatin and chlorhexidine resulted in higher MIC values than for each of the drugs alone [4]. Saurabh S. C. et al., used two antiseptic agents combined with clotrimazole and showed that the combination group of 2% Chlorhexidine with clotrimazole was more effective than 2% chlorhexidine alone[13]. This synergistic effect of chlorhexidine with clotrimazole was in accordance with our study as well. From our disk diffusion data, we infer that even lower concentrations of both drugs result in synergism. We also found that the resistance of clotrimazole by Candida can be reversed by chlorhexidine. From the microdilution chequerboard results, the combination group showed a significant decrease of MICs of both drugs when compared with individual doses. We speculate that low dosages of chlorhexidine acts by binding to the Candida cell membrane resulting in leakage to the Candida cellular contents and thereby increases the penetration of clotrimazole.

Conclusion

By using these synergistic effects, novel formulations including low concentrations of both clotrimazole and chlorhexidine can be designed to have a better effect against superficial or oral and vaginal candidiasis. Further studies on the synergistic mechanisms and in vivo effects are required.

Methods And Materials

Fungal Strains

A total of 25 clinical isolates of Candida spp. (15 strains of C. albicans, 5 strains of Candida glabrata, 2 strains of Candida krusei, and 3 strains of Candida parapsilosis) were collected from Our Hospital. Fungal identification was determined by CHROM agar, API20C AUX and molecular sequencing of the internal transcribed spacer (ITS) ribosomal DNA (rDNA).

Antifungal agents

The tested compounds Clotrimazole (purity ≥ 99%) and Chlorhexidine (purity ≥ 99%) were purchased in powder form from Selleck Chemicals (Houston, TX, USA). The compounds were dissolved in DMSO and prepared as stocks. The working concentration ranges of Clotrimazole and Chlorhexidine were 0.03-16 μg/ml.
In vitro susceptibility test
CLSI microdilution was performed strictly according to CLSI standard M27-A4[10]. Microtiter plates were read visually, and the MIC was determined using prominent inhibition (corresponding to 50%) as an endpoint. C. parapsilosis ATCC 22019 was used as a quality control strain.

Antifungal Combination test
The MIC ranges of individual tested drugs against Candida isolates were >16 µg/ml for Chlorhexidine and 0.5–2 µg/ml for Clotrimazole. Individually, Chlorhexidine at low concentrations, did not exhibit any significant antifungal activity against the tested strains. When Chlorhexidine was combined with Clotrimazole, the MICs of Chlorhexidine and Clotrimazole against Candida spp. decreased to 1–2µg/ml and 0.125–0.5µg/ml, respectively, demonstrating favorable synergistic effects against 21 (84%) strains of Candida spp.

Disk Diffusion
The chlorhexidine disk on its own was ineffective against all the Candida spp. The halos of clotrimazole alone were 7-12mm. On the plates which contained both clotrimazole and chlorhexidine, there was an increase in halo size when compared to clotrimazole alone group. The diameters ranged from 12-18mm (Figure 1, Table 2).

Declarations
Acknowledgement
Not applicable
Competing Interests
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Tables

Table 1 The combination of clotrimazole and chlorhexidine against Candida spp (MIC, ug/ml)
### Table 2

| Strains     | clotrimazole | chlorhexidine | clotrimazole/chlorhexidine | FICI  |
|-------------|--------------|---------------|---------------------------|-------|
| SC5314      | 0.5          | 16            | 0.125/2                   | S     |
| CA1         | 0.5          | 16            | 0.125/1                   | S     |
| CA2         | 0.5          | 16            | 0.125/2                   | S     |
| CA3         | 0.5          | 16            | 0.125/2                   | S     |
| CA4         | 0.5          | 16            | 0.125/2                   | S     |
| CA5         | 0.5          | 16            | 0.125/1                   | S     |
| CA6         | 2            | 16            | 0.5/2                     | S     |
| CA7         | 2            | 16            | 0.5/2                     | S     |
| CA8         | 0.25         | 16            | 0.25/1                    | N     |
| CA9         | 0.25         | 16            | 0.25/1                    | N     |
| CG1         | 0.5          | 16            | 0.125/2                   | S     |
| CG2         | 0.5          | 16            | 0.125/2                   | S     |
| CG3         | 1            | 16            | 0.125/2                   | S     |
| CG4         | 0.5          | 16            | 0.125/2                   | S     |
| CG5         | 1            | 16            | 0.125/1                   | S     |
| CK1         | 1            | 16            | 0.125/2                   | S     |
| CK2         | 0.5          | 16            | 0.125/1                   | S     |
| ATCC22019   | 0.5          | 16            | 0.125/2                   | S     |
| CP1         | 1            | 16            | 0.125/2                   | S     |
| CP2         | 1            | 16            | 0.125/2                   | S     |

**FICI results**: S, synergy (FICI of ≤0.5); N, indifference (no interaction [FICI of >0.5 to ≤4]).

**CA**: *C. albicans*; **CG**: *C. glabrata*; **CK**: *C. krusei*, **CP**: *C. parapsilosis*
| Strains | Clotrimazole 32ug/disk | Chlorhexidine 5ug/disk | clotrimazole/chlorhexidine |
|---------|------------------------|------------------------|---------------------------|
| SC5314  | 11                     | No Effect              | 18                        |
| CA1     | 11                     | No Effect              | 17                        |
| CA2     | 10                     | No Effect              | 15                        |
| CA3     | 12                     | No Effect              | 18                        |
| CA4     | 11                     | No Effect              | 17                        |
| CA5     | 11                     | No Effect              | 18                        |
| CA6     | 12                     | No Effect              | 18                        |
| CA7     | 11                     | No Effect              | 17                        |
| CA8     | 10                     | No Effect              | 15                        |
| CA9     | 11                     | No Effect              | 15                        |
| CG1     | 8                      | No Effect              | 14                        |
| CG2     | 9                      | No Effect              | 15                        |
| CG3     | 9                      | No Effect              | 15                        |
| CG4     | 9                      | No Effect              | 14                        |
| CG5     | 10                     | No Effect              | 15                        |
| CK1     | 7                      | No Effect              | 18                        |
| CK2     | 7                      | No Effect              | 17                        |
| ATCC22019 | 8                   | No Effect              | 14                        |
| CP1     | 7                      | No Effect              | 12                        |
| CP2     | 7                      | No Effect              | 14                        |

Values represent mean ± SD of the haloes observed in three independent experiments.

CA: *C. albicans*; CG: *C. glabrata*; CK: *C. krusei*, CP: *C. parapsilosis*

**Figures**
The combination of clotrimazole (32ug/plate) and chlorhexidine (5ug/plate) against Candida spp. a. SC5314, clotrimazole plate; b. ATCC22019, clotrimazole plate; c. C. krusei, clotrimazole plate; d. SC5314, chlorhexidine plate; e. ATCC22019, chlorhexidine plate; f. C. krusei, chlorhexidine plate; g. SC5314, clotrimazole and chlorhexidine plate; h. ATCC22019, clotrimazole and chlorhexidine plate; i. CK1:C. krusei, clotrimazole and chlorhexidine plate;
