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ORIGINAL ARTICLE

In vitro interactions between fluconazole and minocycline against mixed cultures of *Candida albicans* and *Staphylococcus aureus*

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Background/purpose: It is difficult to manage coinfections in critically ill patients, especially in the presence of mixed-species biofilms. The aim of this study was to seek an effective drug combination for managing the dual-species biofilm of *Candida albicans* and *Staphylococcus aureus*.
Methods: The interaction between fluconazole and minocycline against polymicrobial planktonic cells and polymicrobial biofilms formed over four different time intervals (4 hours, 8 hours, 12 hours, and 24 hours) was investigated using a microdilution checkerboard method. To explore whether the combined effects against the polymicrobial cultures involved calcium regulation, the effects of benidipine and ethylene glycol tetraacetic acid were characterized using a plate streaking method and a liquid-based quantitative method.
Results: Fluconazole combined with minocycline exerted strong effects against polymicrobial planktonic cells and polymicrobial biofilms formed over 4 hours, 8 hours, and 12 hours. The addition of benidipine and ethylene glycol tetraacetic acid enhanced the activity of the drug combination, suggesting that the combined effects may involve the perturbation of calcium homeostasis.

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Conclusion: Fluconazole in combination with minocycline is a potential approach for counteracting *C. albicans*—*S. aureus* dual-species biofilms.

Introduction

Candida albicans is the fourth most common cause of bloodstream infections and is associated with the highest mortality.[1,2] It has been reported that 27% of nosocomial *C. albicans* bloodstream infections are polymicrobial, with *Staphylococcus aureus* being the third most common microorganism isolated with *C. albicans*.[3] Not only are *C. albicans* and *S. aureus* independently responsible for a myriad of monomicrobial infections, but there is also increasing evidence in the literature to suggest that they are commonly associated with polymicrobial infections.[4]

The extensive use of indwelling medical devices offers favorable conditions for biofilm formation, and it has been estimated that biofilms account for $>$ 80% of all infections in the human body.[5] *C. albicans* has a powerful ability to develop biofilms on biomaterials, and *S. aureus* preferentially adheres to *C. albicans* hyphae, which can result in the formation of *C. albicans*—*S. aureus* polymicrobial biofilms.[6] Biofilm-embedded microorganisms possess notorious resistance to many antimicrobial agents and the ability to resist the immune response in the body. Furthermore, the organisms within polymicrobial biofilms exhibit uniquely altered gene expression, amplified pathogenic phenotypes, and altered antibiotic susceptibility during multispecies interactions.[7–9] For example, *C. albicans* in combination with *S. aureus* produces synergism and increased morbidity and mortality in mice.[10,11] Antimicrobial selection for infections caused by polymicrobial biofilms presents a challenge to clinical management because polymicrobial biofilms are more complicated and difficult to eradicate than monomicrobial biofilms. Therefore, there is an urgent need to explore management options for polymicrobial biofilms. Considerable effort has been made to counteract biofilms, and drug combination therapy is one effective approach.[12,13] The aim of this study was to seek an effective drug combination for the management of *C. albicans*—*S. aureus* polymicrobial biofilms.

Fluconazole is the most common first-line drug for the treatment and prevention of *C. albicans* infections. However, due to its widespread use in clinical settings, an increasing number of fluconazole-resistant strains are being isolated. Similarly, an increasing prevalence of methicillin-resistant *S. aureus* (MRSA) has been observed over recent years, and MRSA can result in serious healthcare-associated infections.[14,15] Our previous research has demonstrated that fluconazole can work synergistically with minocycline against fluconazole-resistant *C. albicans*, and minocycline enhances the amount of fluconazole that penetrates *C. albicans* biofilms.[16] This observation has prompted us to consider whether the combination of fluconazole and minocycline could have a strong effect on *C. albicans*—*S. aureus* polymicrobial biofilms. In the present study, the effects of fluconazole in combination with minocycline were investigated when used against dual-species planktonic and biofilm cultures in addition to single-species cultures. Additionally, the impact of calcium regulation on the drug combination against dual-species cultures was investigated to provide data for future research into its mechanism of action.

Methods

Fungal and bacterial isolates and suspension preparation

Four *C. albicans* clinical isolates (2 azole-susceptible strains, CA8/CA14, plus 2 azole-resistant strains, CA10/CA137) and four *S. aureus* isolates (2 oxacillin-susceptible strains, SA4/SA5, plus 2 oxacillin-resistant strains, SA6/SA10) were used. The susceptibility of these strains was determined according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M100-S19 guidelines with *C. albicans* ATCC 10231 and *S. aureus* ATCC 25923 as reference strains.[17,18] All eight strains were tested in this study, and they were identified and then confirmed. Frozen stocks of the isolates were maintained at −70°C.

Prior to each experiment, all *Candida* and *Staphylococcus* strains were subcultured at least twice at 35°C on their respective culture media, with fungi on yeast—peptone—dextrose (YPD) agar medium containing 1% (w/v) yeast extract, 2% peptone, 2% dextrose, and 2% agar and bacteria on nutrient agar medium containing 1% peptone, 0.3% beef extract, 0.5% NaCl, and 1.5% agar. After ~18 hours, the fungal and bacteria cells were harvested and suspended in sterile phosphate-buffered saline (pH 7.2). The concentration of the *Candida* suspension was measured using a hemocytometer, and *Staphylococcus* cells were counted using the live/dead Baclight staining method.

Preparation of drug stock solutions

Fluconazole was kindly provided by Cheng Chuang Pharmaceutical Co. Ltd., Jinan, China, and minocycline and benidipine were kindly provided by An Bei Ka Pharmaceutical Co. Ltd., Jinan, China. Stock solutions of fluconazole and benidipine were prepared in sterile distilled water and dimethyl sulfoxide, respectively, at a concentration of 2560 μg/mL, and minocycline was prepared in dimethyl sulfoxide at a concentration of 6400 μg/mL. All stock solutions were stored at −70°C until use.

Determination of minimal inhibitory concentrations against dual-species planktonic cultures of *C. albicans* and *S. aureus*

As a result of preliminary experiments with a range of media, RPMI 1640 medium (GIBCO, Life Technologies, The

Netherlands) buffered with 0.165 M morpholinepropanesulfonic acid (Ding Guo Biological Co. Ltd., Beijing, China) was selected as the liquid medium that was best able to support the growth of both species. One of the four *C. albicans* isolates was combined with one of the four *S. aureus* isolates to create eight groups of dual-species cultures as described in Table 1. The mixed-species cells were regarded as an integrality, and a checkerboard broth microdilution assay was used to determine the minimum inhibitory concentrations (MICs) of fluconazole and minocycline alone and in combination against planktonic cells. In accordance with CLSI M27-A3, serial twofold dilutions were performed for each drug, and 50 μL of each drug dilution was added.[17] The final concentration of fluconazole ranged from 0.25 μg/mL to 256 μg/mL, and minocycline ranged from 1 μg/mL to 64 μg/mL in the plate. The *C. albicans* and *S. aureus* suspensions were diluted and mixed to obtain twice the final inoculum of 1×10^3 colony-forming units (CFU)/mL for *C. albicans* and 1×10^5 CFU/mL for *S. aureus*, and 100 μL of the mixed-species inoculum was added to 96-well plates. The plates were incubated at 35°C for 48 hours. After 48 hours, a reagent containing 12.5 parts XTT (2,3- bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) to one part menadione was added to each well followed by incubation for 2 hours in the dark. Colorimetric changes were measured at 492 nm with a microtiter plate reader (Multiskan MK3; Thermo Labsystems). The MIC was defined as the lowest drug concentration that produced an 80% reduction in absorption compared with that of the drug-free control well. The experiments were performed in triplicate on different days.

Determination of sessile MICs against dual-species biofilms of *C. albicans* and *S. aureus*

The checkerboard microdilution assay was also used to determine the sessile MICs (SMICs) of fluconazole and minocycline alone and in combination against polymicrobial biofilms. The same eight polymicrobial culture models above were used to form 4-hour, 8-hour, 12-hour, and 24-hour polymicrobial biofilms on 96-well flat-bottomed plates at 35°C. The biofilm-forming concentration was 1×10^3 CFU/mL for *C. albicans* and 1×10^5 CFU/mL for *S. aureus*. After the formation of biofilms, the medium was aspirated, and each well was washed three times with sterile phosphate-buffered saline to remove loosely adhered cells, followed by the addition of 100 μL fresh medium. Subsequently, each serially diluted drug was added, bringing the final concentration of fluconazole in the wells to a range of 0.5—512 μg/mL and that of minocycline to a range of 2—128 μg/mL. The whole system was incubated for 48 hours at 35°C. After 48 hours, the biofilms were quantified using the XTT reduction assay.[19] The definition of SMIC was the same as that of MIC. Each experiment was performed in triplicate on different days.

Effects of calcium regulation on drug interactions

The effects of benidipine and ethylene glycol tetraacetic acid (EGTA) on the drug combination against the dual-species cultures were preliminarily investigated using the plate streaking method. A culture medium composed of 0.25% (w/v) yeast extract, 1.25% peptone, 0.5% dextrose, 0.375% NaCl, 0.25% beef, and 1.625% agar was selected as the solid medium best able to support the growth of both species as shown in Fig. 1. The final concentrations were 2 μg/mL fluconazole, 8 μg/mL minocycline, 8 μg/mL benidipine, and 0.5 mM for EGTA. For qualitative evaluation, four *Candida* strains with different susceptibilities were combined with one *Staphylococcus* strain to create four polymicrobial culture groups. The polymicrobial suspension (1.5×10^6 CFU/mL yeast and 1.5×10^8 CFU/mL *Staphylococcus*) was coated onto the delimited area of the plate. The plates were incubated at a constant temperature of 35°C for 48 hours, after which the growth of the mixed cultures was observed visually.

To quantify the effects of benidipine and EGTA on the drug combination, the microdilution assay in 96-well plates was performed. The effects of benidipine (0 μg/mL, 4 μg/mL, and 8 μg/mL) and EGTA (0 mM, 0.25 mM, and 0.5 mM) on the two groups of drug combinations composed of different concentrations of fluconazole and minocycline were evaluated against dual-species planktonic and biofilm cultures of CA10 and SA12. Benidipine/EGTA alone and fluconazole in combination with minocycline were used as a control. The concentrations of fluconazole and minocycline in the two groups ranged from low to high. The planktonic and biofilm dual-species cells were prepared as described above. The 96-well plates were incubated for 48 hours at 35°C. The viability of the mixed-species cells was determined using the XTT reduction assay as described above. For the three independent experiments, one-way analysis of variance followed by the Newman—Keuls multiple comparison test were used to determine the differences between different treatment groups at the chosen significance level ($p < 0.05$).

Table 1 Combined drug effects against polymicrobial planktonic cells

Polymicrobial PC[a]	MIC[b] (μg/mL)			
	Alone		In combination	
	FLC	MINO	FLC	MINO
R_{CA137}-R_{SA10}	>256	>64	2	4
R_{CA137}-S_{SA4}	>256	>64	1	4
S_{CA8}-R_{SA10}	>256	>64	0.5	2
S_{CA8}-S_{SA4}	>256	>64	0.5	1
R_{CA10}-R_{SA12}	>256	>64	4	4
R_{CA10}-S_{SA5}	>256	>64	2	1
S_{CA14}-R_{SA12}	>256	>64	4	4
S_{CA14}-S_{SA5}	>256	>64	0.5	1

[a] Polymicrobial planktonic *Candida albicans* and *Staphylococcus aureus* cells.
[b] $MIC_{80\%}$ of each drug alone or in combination against polymicrobial planktonic *C. albicans* and *S. aureus* cells shown as the median of three independent experiments.
CA = *C. albicans*; FLC = fluconazole; MIC = minimum inhibitory concentration; MINO = minocycline; R = resistant strain; R_{CA}-R_{SA} = polymicrobial cultures of FLC-resistant *C. albicans* and oxacillin-resistant *S. aureus*; S = susceptible strain; SA = *S. aureus*.

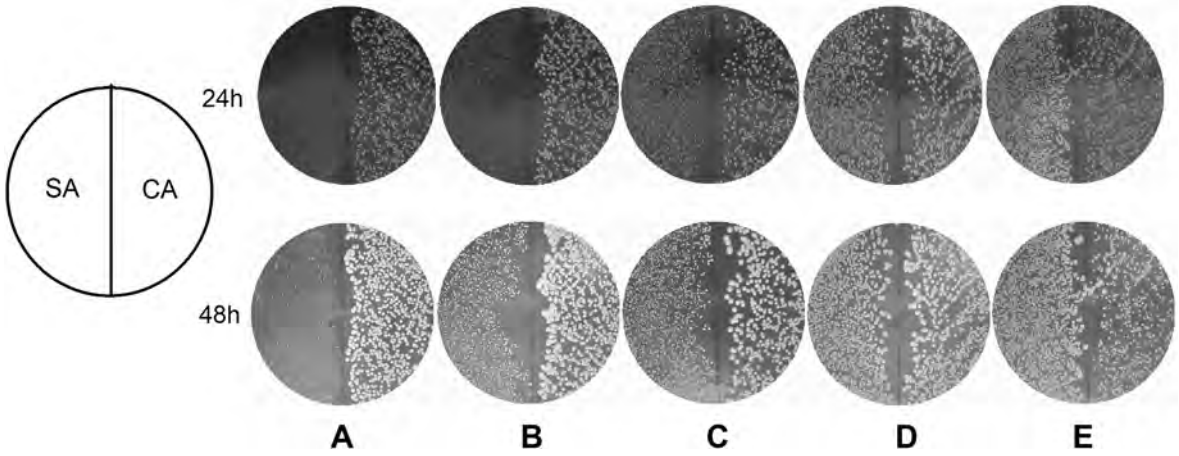

Figure 1. Propagation conditions for *Staphylococcus aureus* (left) and *Candida albicans* (right) on different culture media. By comprehensively comparing strain growth after incubation for 24 hours and 48 hours at 35°C, the most vigorous propagation occurred in Group D. A—E: different nutritional ingredient groups. Group A: 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar. Group B: 0.75% yeast extract, 1.75% peptone, 1.5% dextrose, 0.125% NaCl, 0.075% beef, and 1.875% agar. Group C: 0.5% yeast extract, 1.5% peptone, 1% dextrose, 0.25% NaCl, 0.15% beef, and 1.75% agar. Group D: 0.25% yeast extract, 1.25% peptone, 0.5% dextrose, 0.375% NaCl, 0.25% beef, and 1.625% agar. Group E: 1% peptone, 0.5% NaCl, 0.3% beef, and 1.5% agar.

Results

In vitro combined effects of fluconazole and minocycline against dual-species planktonic cultures

The $MIC_{80\%}$s of fluconazole against *C. albicans* CA8, CA10, CA14, and CA137 were 0.25 μg/mL, 512 μg/mL, 0.5 μg/mL, and 64 μg/mL, respectively, and 0.5 μg/mL, 0.5 μg/mL, 4 μg/mL, and 4 μg/mL, for minocycline against *S. aureus* SA4, SA5, SA10, and SA12, respectively. The MICs of the two quality control strains fell within the normal range. The combination of fluconazole and minocycline produced synergistic effects against fluconazole-resistant *C. albicans*, whereas fluconazole did not potentiate the effect of minocycline against *S. aureus*. Each drug alone did not produce obvious effects against the dual-species planktonic cultures, whereas the combination of both produced strong effects. The results are summarized in Table 1. As shown in Table 1, the addition of minocycline resulted in a decrease in the MIC of fluconazole from 512 μg/mL to 2—4 μg/mL against the mixed fungal-bacterial culture containing *C. albicans* CA10.

In vitro combined effects of fluconazole and minocycline against dual-species biofilms

Minocycline potentiated the effect of fluconazole against *C. albicans* biofilms, whereas fluconazole did not enhance the effect of minocycline against *S. aureus* biofilms. Fluconazole and minocycline alone had little effect on the dual-species biofilms, whereas fluconazole in combination with minocycline had a potent effect on all of the polymicrobial biofilms formed for 4 hours, 8 hours, and 12 hours. The combined effects against the 4-hour and 8-hour biofilms were similar to those against planktonic cells (data not shown). The results of the experiments involving the 12-hour biofilms are summarized in Table 2. The combination of 8 μg/mL fluconazole and 8 μg/mL minocycline produced approximately 80% inhibition of the growth of biofilms formed by fluconazole-resistant *C. albicans* CA10 and oxacillin-resistant *S. aureus* SA12 compared with the control well. However, no obvious effect was observed against the 24-hour biofilms.

Impact of calcium regulation on drug interactions

The results of a preliminary investigation are shown in Fig. 2. Compared with the growth control group, cotreatment with fluconazole and minocycline inhibited the

Table 2 Combined drug effects against the polymicrobial biofilms

Polymicrobial BF[a]	SMIC[b] (μg/mL)			
	Alone		In combination	
	FLC	MINO	FLC	MINO
R_{CA137}-R_{SA10}	>512	>128	4	8
R_{CA137}-S_{SA4}	>512	>128	2	8
S_{CA8}-R_{SA10}	>512	>128	1	8
S_{CA8}-S_{SA4}	>512	>128	1	2
R_{CA10}-R_{SA12}	>512	>128	8	8
R_{CA10}-S_{SA5}	>512	>128	4	4
S_{CA14}-R_{SA12}	>512	>128	8	8
S_{CA14}-S_{SA5}	>512	>128	1	2

[a] Polymicrobial biofilms formed for 12 hours.
[b] $SMIC_{80\%}$ of each drug alone or in combination against 12-hour polymicrobial *C. albicans*—*S. aureus* biofilms shown as the median of three independent experiments.
CA = *C. albicans*; MINO = minocycline; R = resistant strain; R_{CA}-R_{SA} = polymicrobial cultures of FLC-resistant *C. albicans* and oxacillin-resistant *S. aureus*; S = susceptible strain; SA = *S. aureus*; SMIC = sessile minimum inhibitory concentration.

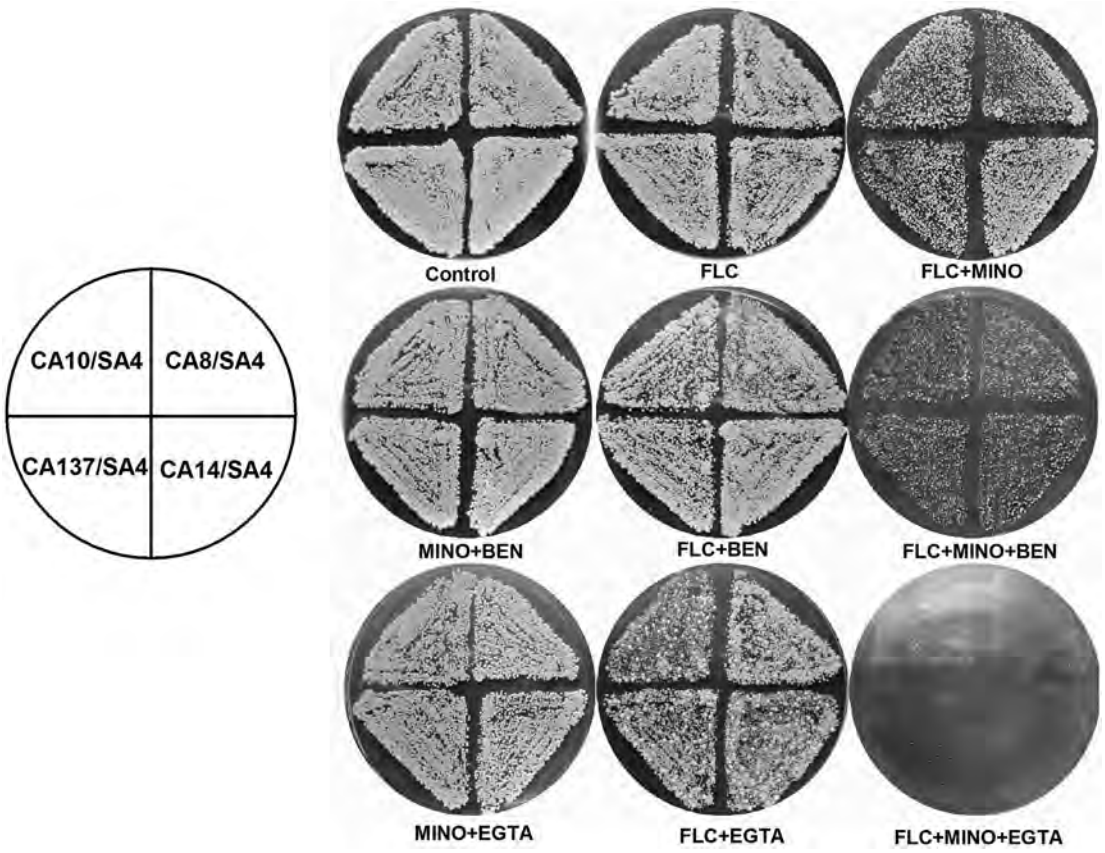

Figure 2. Effects of BEN/EGTA on the combination of FLC and MINO against mixed cultures of *Candida albicans* CA10-SA4, CA137-SA4, CA8-SA4, and CA14-SA4 after incubation for 48 hours. The framework on the left characterizes the arrangement of the mixed species of *C. albicans* and *Staphylococcus aureus* in the culture dish. The concentrations of FLC, MINO, BEN, and EGTA in the plates were 2 μg/mL, 8 μg/mL, 8 μg/mL, and 0.5 mM, respectively. BEN = benidipine; EGTA = ethylene glycol tetraacetic acid; FLC = fluconazole; MINO = minocycline.

growth of *C. albicans* and *S. aureus*, but the presence of benidipine or EGTA increased the effectiveness of the drug combination. The potentiation caused by the addition of EGTA appeared to be stronger than that caused by the addition of benidipine, with the combination of fluconazole, minocycline, and EGTA almost completely inhibiting growth.

The results of the quantitative liquid assays indicated that the addition of benidipine/EGTA produced large increases in percent growth inhibition compared with the control groups (Fig. 3), and the higher the concentration of the added benidipine/EGTA, the greater the effect against planktonic cells. The addition of 0.25/0.5 mM EGTA to the combination of fluconazole and minocycline produced a statistically significant difference, whereas the addition of 4/8 μg/mL benidipine did not. The same phenomenon was observed for 8-hour and 12-hour dual-species biofilms, but for 24-hour biofilms, adding benidipine and EGTA did not reduce growth (data not shown).

Discussion

Most previous research has focused on single-species bacterial or fungal biofilms, and little research has involved mixed fungal—bacterial biofilms. Furthermore, the current research associated with polymicrobial biofilms is primarily dedicated to determining how fungi and bacteria interact in biofilms.[20,21] Few studies have investigated drug intervention against polymicrobial biofilms. The results of the present study indicate that fluconazole in combination with minocycline are highly effective against dual-species planktonic and biofilm cultures of *C. albicans* and *S. aureus*. Fluconazole in combination with minocycline produced a synergistic effect on resistant *C. albicans* planktonic cells and their biofilms, whereas fluconazole did not enhance the effect of minocycline against *S. aureus* cultures. The potent efficacy of the drug combination against the two species may be explained by the fact that minocycline can potentiate the effect of fluconazole against *C. albicans* and that minocycline itself can inhibit the growth of *S. aureus*.

In this study, a new medium for culturing the mixed species was explored, and the optimal components and proportion of the nutritional ingredients may be useful to researchers in this field. As for the dual-species biofilms that formed for no more than 12 hours, the combined effect of fluconazole and minocycline was potent. However, no obvious effect against the 24-hour biofilms was observed. The anti-biofilm effects may be attributed to the

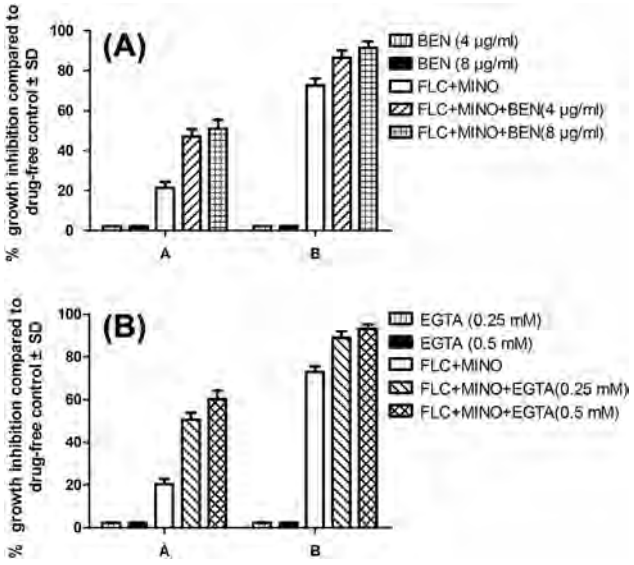

Figure 3. Effects of (A) BEN and (B) EGTA on the two drug combination groups composed of different concentrations of FLC and MINO against polymicrobial planktonic *C. albicans* CA10 and SA12 cells. The bar chart denotes the percent growth inhibition of different drug combinations against the mixed-species cultures. In Group A, the concentration of both FLC and MINO was 1 μg/mL. In Group B, the concentration of both FLC and MINO was 2 μg/mL. In each sample containing FLC and MINO and BEN or EGTA, the effect was significant ($p < 0.01$) compared with the control samples (the first three columns). Group B: Addition of 0.25/0.5 mM EGTA to the combination of FLC and MINO produced a significant difference, with $p < 0.01$ in Group A and $p < 0.05$ in Group B. BEN = benidipine; EGTA = ethylene glycol tetraacetic acid; FLC = fluconazole; MINO = minocycline.

fact that the combination of fluconazole and minocycline inhibits the formation of polymicrobial biofilms. This drug combination may serve as an effective approach against polymicrobial biofilms, including those formed by resistant strains.

Calcium homeostasis is crucial for the normal growth of *C. albicans*.[22] Our previous study demonstrated that one of the predominant mechanisms by which minocycline potentiates fluconazole against *C. albicans* involves interrupting the cellular calcium balance.[16] The results of this work demonstrate that the addition of benidipine or EGTA enhances the activity of the drug combination, and the effect is stronger for EGTA than for benidipine. Moreover, no obvious difference was observed in the quantitative liquid assays, which may be attributed to the different methods used and the different concentrations of the mixed-species suspension. These results suggest that the mechanism of action of the drug combination involves calcium dysregulation.

In conclusion, the combination of fluconazole and minocycline produced a strong effect against mixed planktonic cultures and biofilms of *C. albicans* and *S. aureus*. The activity of the drug combination was enhanced by benidipine and EGTA, which may provide a basis for future mechanistic studies involving Ca^{2+}.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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