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Strong synergism of dexamethasone in combination with fluconazole against resistant *Candida albicans* mediated by inhibiting drug efflux and reducing virulence

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ABSTRACT

Candida albicans is the most commonly isolated *Candida* spp. in the clinic and its resistance to fluconazole (FLC) has been emerging rapidly. Combination therapy may be a potentially effective approach to combat drug resistance. In this study, the combination antifungal effects of dexamethasone (DXM) and FLC against resistant *C. albicans* in vitro were assayed using minimum inhibitory concentrations (MICs), sessile MICs and time–kill curves. The in vivo efficacy of this drug combination was evaluated using a *Galleria mellonella* model by determining survival rate, fungal burden and histological damage. In addition, the impact of DXM on efflux pump activity was investigated using a rhodamine 6G assay. Expression of *CDR1*, *CDR2* and *MDR1* was determined by real-time quantitative PCR, and extracellular phospholipase activity was detected by the egg yolk agar method to reveal the potential synergistic mechanism. The results showed that DXM potentiates the antifungal effect of FLC against resistant *C. albicans* strains both in vitro and in vivo, and the synergistic mechanism is related to inhibiting the efflux of drugs and reducing the virulence of *C. albicans*.

1. Introduction

Candida spp. are common opportunistic fungal pathogens, among which *Candida albicans* is the most common [1]. Azoles are first-line antifungal drugs for the treatment of *Candida* infections [2]. With the widespread use of antifungal drugs, fluconazole (FLC)-sensitive *Candida* have started to become FLC-resistant [3]. Thus, there is an imminent need to search for new antifungal agents and approaches to treating *C. albicans* infections. Incidentally, the development of new antifungal drugs requires a huge economic investment and time. Therefore, increasingly more attention has been paid to drug combinations, of which the combination of antifungals with non-antifungals has been the focus of several recent studies [4,5].

The treatment of severe infections and septic shock with glucocorticoids has been the subject of debate for many years [6]. In recent years, owing to our in-depth understanding of the pathogenesis of septic shock, increasingly more clinical and basic research has discovered that glucocorticoids are beneficial for the treatment of septic shock [7,8]. The activity of glucocorticoids against bacterial micro-organisms has prompted us to consider whether they can potentiate the antifungal effects of FLC against FLC-resistant *C. albicans* strains.

Dexamethasone (DXM) is a type of glucocorticoid and its main pharmacological actions include anti-inflammatory, antiviral, anti-allergic and antirheumatic effects. In addition, DXM is widely used in the clinic for severe infections and septic shock [9]. In cases of severe infection and septic shock, application of glucocorticoids will be inevitable in order to save the patient's life. In this study, we aimed to examine whether DXM can work synergistically in combination with antifungal drugs. The in vitro and in vivo activities of DXM combined with FLC against *C. albicans* were evaluated and the potential synergistic mechanism was explored.

2. Materials and methods

2.1. Strains and media

Candida strains used in this study included FLC-resistant isolates CA10 and CA16 and FLC-susceptible isolates CA4 and CA8. All of the strains were collected from the clinical laboratory of Qianfoshan Hospital (Jinan, China). *Candida albicans* reference strain ATCC 10231 was obtained from the Pharmacological Institute of

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Shandong University (Jinan, China). Strains were stored at –80 °C, were refreshed and were incubated on YPD (yeast–peptone–dextrose) solid medium (Dalian Meilun Biological Technology, Dalian, China) at least twice at 35 °C before each experiment.

2.2. Drugs

FLC was purchased from Cheng Chuang Pharmaceutical (Jinan, China) and was dissolved in water to a concentration of 2560 μg/mL. DXM was provided by the Chinese Medicine Institute (Beijing, China) and stock solutions of 2560 μg/mL were prepared in anhydrous ethanol.

2.3. In vitro antifungal effects of dexamethasone combined with fluconazole

2.3.1. Antifungal susceptibility testing

The in vitro antifungal activities of DXM and FLC were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (document M27-A3). For chequerboard experiments, a microtitre plate containing RPMI medium in the absence and presence of different concentrations of DXM and FLC, alone or in combination, were used. In brief, serial doubling dilutions of the tested drugs were prepared (0.0625–32 μg/mL for FLC and 2–128 μg/mL for DXM). Either 50 μL of drug or mixture was added to each well. Then, 100 μL of a *C. albicans* CA10 cell suspension (2×10^3 cells/mL) was added to each of the wells.

All of the wells were filled with RPMI 1640 to a final volume of 200 μL. Plates were then incubated at 35 °C for 24 h [10] and the results were obtained by measuring the optical density at 492 nm. Minimum inhibitory concentration (MIC) endpoints were defined as the lowest concentration of drug, alone or in combination, that inhibited the growth of yeast (80% inhibition of growth compared with the control). All experiments were performed in triplicate.

Determination of drug interactions was based on the fractional inhibitory concentration index (FICI) model and the percentage of growth difference (ΔE) model, respectively [11–13]. A FICI ≤ 0.5 represents synergy, an FICI > 4.0 represents antagonism, and an FICI > 0.5 or ≤ 4.0 represents no interaction [14]. The ΔE model was defined by the following equation: $\Delta E = E_{predicted} - E_{measured}$, with $E_{measured}$ obtained directly from the experimental data. The following equation was derived: $E_{predicted} = E_A \times E_B$, where E_A and E_B are the experimental percentages of fungal growth of each drug action alone, respectively. Statistically significant interactions <100% were considered weak, interactions between 100% and 200% were considered moderate, and interactions >200% were considered strong, as described previously [15].

2.3.2. Antibiofilm susceptibility testing

Biofilms were formed as described previously [16]. Briefly, biofilms were formed over four time intervals (4, 8, 12 and 24 h) at 35 °C. An aliquot of 100 μL of the standardised cell suspension (1×10^3 CFU/mL) was added into a 96-well plate. At each time point, biofilms were washed three times with sterile phosphate-buffered saline (PBS) to remove planktonic yeast cells and then FLC and DXM were added to the 96-well plate. The final concentration of FLC in wells ranged from 1–512 μg/mL and that of DXM ranged from 4–256 μg/mL. Control wells were filled with RPMI 1640 without antifungal agents. Plates were incubated at 35 °C for 24 h. A colorimetric reduction assay was performed with XTT according to the protocol of Melo et al [17]. Absorbance was measured using a SpectraMax 190 microtitre plate reader (Thermo Lab Systems, Wisconsin) at 492 nm.

2.3.3. Time–kill curves study

A time–kill curve (CFU as a function of time) was used to examine the rate and extent of reduction in *C. albicans* burden when treated with DXM and FLC alone or in combination. A concentration of 1×10^3 cells/mL of *C. albicans* CA10 was used in this experiment. The final concentration was 1 μg/mL of FLC when combined with 32 μg/mL of DXM. A drug-free sample served as a growth control. The XTT test was performed to detect cell viability after different treatments as described previously [18,19].

In brief, samples were collected at predetermined time points (0, 6, 12, 24 and 48 h) following incubation at 35 °C. A 100 μL aliquot was taken from each treatment mixture and was transferred into the wells of a new 96-well microtitre plate; then, 100 μl of XTT/menadione solution was added. For the XTT/menadione solution, prior to each assay, XTT (Sigma, St Louis, MO) was dissolved in a saturated solution at 0.5 μg/mL in Ringer's lactate. The solution was then filter-sterilised through a 0.22 μm filter and 100 mM menadione in acetone was added to a final concentration of 10 μM. The plate was incubated in the dark for 2 h at 35 °C. Afterwards, the absorbance was measured at 492 nm on a SpectraMax 190 microplate reader. All experiments were performed in triplicate and the final results were reported as the mean value.

2.4. In vivo antifungal effects of dexamethasone combined with fluconazole

2.4.1. Galleria mellonella survival assay

Greater wax moth (*G. mellonella*) larvae killing assays were performed as described previously [20]. In brief, groups of 22 larvae (0.3–0.6 g) were each inoculated with 10 μL of a 1×10^8 yeast cells/mL suspension of *C. albicans* CA10 in PBS buffer containing 20 μg/mL cefazolin to prevent bacterial contamination. Thus, the final inoculum concentration for each group was 1×10^6 yeast cells/larva. The final concentration was 160 μg/mL both for FLC and DXM. A drug-free sample served as a control. Yeast suspensions were injected into the haemocoel through the last left proleg of the larva using a 10 μL syringe. Infected larvae were treated with 10 μl of FLC, DXM, a mixture of FLC + DXM, or PBS and were incubated at 37 °C. Death was monitored daily for 4 days. The larvae were considered dead when they did not respond to physical stimulation (slight pressure with forceps). Each experiment was repeated at least three times and representative experiments are presented.

2.4.2. Fungal burden study

Fungal burden was determined by the colony counting method at different times following inoculation. For this purpose, four groups of 20 larvae were selected, all of which were inoculated with 1×10^6 cells/larva of the *C. albicans* strain CA10. One group remained untreated, whilst two other groups were treated with FLC 160 μg/mL or DXM 160 μg/mL, and the last group was treated with FLC 160 μg/mL combined with DXM 160 μg/mL. Once inoculated and treated, two larvae were taken from each group, were washed with ethanol and were cut into small pieces with a scalpel. The material was suspended in 2 mL of PBS/ampicillin and was homogenised gently with a vortex for a few seconds. The homogenate was diluted 10-fold with the same buffer. Then, 10 μL of each sample was inoculated onto Sabouraud (Oxoid Ltd., Basingstoke, UK)/chloramphenicol (Sigma) agar plates. Plates were incubated for 24 h at 35 °C and CFUs were enumerated, with the results expressed as the mean ± standard deviation [21].

2.4.3. Histological study

To evaluate the presence of *C. albicans* inside tissue of *G. mellonella*, three larvae per group were fixed for 24 h in 4% buffered formalin