Efficacy of a Fixed Combination of Tetracycline, Chloramphenicol, and Colistimethate Sodium for Treatment of *Candida albicans* Keratitis

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Purpose. To evaluate the antifungal activity of a fixed antibiotic combination (AC) containing tetracycline (TET), chloramphenicol (CAF), and colistimethate sodium (CS).

Methods. In vitro: *Candida* ATCC and clinical strains were used. The minimum inhibitory concentrations (MICs) of AC and of each antibiotic were determined. Fluconazole (FLC) was tested for comparison. Time-killing curves of selected strains were performed. Ex vivo keratitis: corneas were injected intrastromally with the selected strains. After the injection, corneas were divided into groups of treatments: AC, FLC, or saline. Then, the tissues were analyzed for colony-forming units per gram (CFU/g). Propidium iodide (PI) and MitoTracker (MTR) staining were used to investigate the mode of action.

Results. Values of MIC required to inhibit the growth of 90% of organisms for the antibiotics alone were higher than FLC. However, their activity was enhanced when used in combination against *Candida* yeasts. Time-killing curves showed that at 24 hours, AC reduced the load of both strains of approximately 1 Log_{10} CFU/g compared with the initial inoculum ($P < 0.0001$). This effect was also significant versus FLC. In ex vivo, AC was effective in decreasing the loads of both strains by 4 Log_{10} CFU/g with respect to the control. Moreover, it showed higher activity than FLC against *Candida albicans* ATCC 10231 (1 Log_{10} CFU/g, $P < 0.01$ versus control). PI staining demonstrated that CS changed the membrane's permeability, whereas MTR staining demonstrated that TET or CAF altered mitochondrial function. The cells treated with AC and stained showed both effects.

Conclusions. In this study, AC showed antifungal efficacy versus *Candida* spp.; this activity can be due to the synergistic effects of antibiotics in it.

Keywords: *Candida*, tetracycline, chloramphenicol, colistimethate sodium, fungal keratitis, mode of action

Mycotic keratitis, commonly known as fungal keratitis, accounts for approximately 1% to 44% of all cases of microbial keratitis, depending on the geographic location.[1,2] The genera that commonly cause infection of the cornea include *Fusarium*, *Aspergillus*, *Curvularia*, *Bipolaris*, and *Candida*.[1–3] Among the *Candida* species, *Candida albicans* is the most common etiologic agent of keratitis.[4] In this form of keratitis, one or more ocular (e.g., insufficient tear secretion, defective eyelid closure) or systemic (e.g., diabetes mellitus, immunosuppression) conditions predispose to the infection. This form of mycotic infection also may supervene on a preexisting epithelial defect due to herpes keratitis or due to abrasions caused by contaminated contact lenses.[5]

Management of fungal keratitis largely involves a decision on which antifungal to use and the route of administration. Most of the currently available antifungal medications have limitations, such as poor bioavailability and limited ocular penetration, especially in cases with deep-seated lesions.[6–8] These factors, particularly especially in cases of severe fungal keratitis, account for the slow resolution of fungal infections, with most cases finally requiring a therapeutic penetrating keratoplasty (PKP).[8]

Clinically, the commercially available Natamycin 5% suspension is the initial drug of choice for fungal keratitis. If worsening of the keratitis is observed on topical Natamycin, Amphotericin B (amp B) can be substituted, although topical azoles (e.g., fluconazole [FLC] and voriconazole) are considered to be a good alternative to amp B for the treatment of *Candida* keratitis. They have better ocular penetration and are less toxic to the corneal epithelium, compared with amp B.[9–11]

The clinician must determine the length of treatment for each case based on clinical response and experience. Treatment with a systemic antifungal agent is recommended in cases of severe deep keratitis, scleritis, and endophthalmitis. Systemic antifungals are also used after PKP for fungal keratitis.[9]

Improvement of the antifungal arsenal is needed because existing antifungals can be associated with limited efficacy, toxicity, and resistance.[12] The emergence of resistant fungal strains to current antifungals, which is exacerbated by the

necessity for long-term usage of antifungal in immunocompromised individuals, causes additional difficulty in treatment.[13]

Recent scientific studies have reevaluated old antibiotics, such as chloramphenicol, tetracyclines, and polymyxins, traditionally used for bacterial infections, for their potential antifungal activity.[14–17]

Based on literature and clinical experience, in this study, we evaluated the antifungal activity of a widely used antibacterial ophthalmic combination (Colbiocin; SIFI SpA, Catania, Italy) containing tetracycline (TET), chloramphenicol (CAF), and colistimethate sodium (CS), using in vitro tests and an ex vivo mycotic keratitis model. Specific assays were also carried out to understand the mechanism of action.

Materials and Methods

Antimicrobial Agent

Fixed antibiotic combination (AC) (Colbiocin; SIFI SpA) contained CAF (4 mg/mL), TET (5 mg/mL), and CS (14.4 mg/mL). Fluconazole (FLC) was obtained from Sigma-Aldrich, Milan, Italy; CAF from Química Sintética S.A., Madrid, Spain; TET from Ningxia Qiyuan Pharmaceutical Co., Ningxia, China; and CS from Xellia Pharmaceuticals APS Dalslandsgade, Copenhagen, Denmark.

Strains

The following strains, obtained from Italian hospitals in Messina and Catania, were used for the antimicrobial testing: *Candida albicans* ATCC 2091, *C. albicans* ATCC 10231, and 14 clinical isolates of *C. albicans* ($n = 7$), *Candida glabrata* ($n = 5$), *Candida utilis*, and *Candida tropicalis*. The yeasts were stored at –70°C in Microbanks vials (DID; Pro-Lab Diagnostics, Ontario, Canada).

In Vitro Study

Antifungal Susceptibility Testing. Drug susceptibility was determined using the Clinical and Laboratory Standards Institute microbroth dilution protocol.[18,19] Cultures for antifungal activity tests were grown in RPMI-1640 medium supplemented with MOPS (Oxoid, Milan, Italy) at 30°C (48 hours).

Working cultures of yeasts were adjusted to the required concentration of 10^3 colony-forming units per milliliter (CFU/mL). The AC power was reconstituted in 5 mL buffer containing EDTA, Polysorbate 80, and purified water. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to dissolve CAF and TET and then diluted to the highest concentration (1% vol/vol) using RPMI-1640 medium. FLC was dissolved in RPMI-1640 medium. Serial doubling dilutions of the AC and antimicrobial agents were prepared in 96-well microtiter plates over the range of 0.016 to 2 mg/mL in RPMI-1640 medium supplemented with MOPS. The plates were incubated for 48 hours at 35°C. Growth controls (medium with inocula) were included. MIC_{90} is defined as the minimum inhibitory concentration required to inhibit the growth of 90% of organisms. To determine MFC (defined as the lowest concentration of antimicrobial agents at which 99.9% of microorganisms were killed), broth was taken from each well and inoculated in Sabouraud Dextrose Agar (SDA) for 48 hours at 35°C. Each experiment was performed in triplicate.

Checkerboard. The checkerboard broth microdilution assay, a two-dimensional array of serial concentrations of test compounds, was used to assess drug synergism. The minimum inhibitory concentrations (MICs) of each antibiotic included in the AC were determined either individually or in combination for two selected strains: *C. albicans* ATCC 10231 and *C. albicans* n. 4 clinical isolate. FLC was tested for comparison. Yeast suspensions were prepared to yield final inoculum of approximately 2×10^3 CFU/mL. Microplates were read after 48/72 hours of incubation at 35°C. Each test was performed in triplicate. The results were analyzed using the fractional inhibitory concentration index (FICI), which was calculated as follows: FICI = (FICA + FICB [or FICC]), where FICA = (MIC of compound A in the presence of compound B)/(MIC of compound A alone). Similarly, the fractional inhibitory concentration for compound B (or compound C) was calculated. An FICI value of ≤ 0.5 was interpreted as synergy, whereas the FICI values between 0.5 and 1.0 were interpreted as additive. FICI values >4.0 were considered as antagonism and FICI values between 1.0 and 4.0 were considered as indifferent.[20]

Time-Killing Curve. Time-killing curves for *C. albicans* ATCC 10231 and *C. albicans* n. 4 clinical isolate were performed at 10 times MIC values of AC and FLC. Yeast suspensions were prepared to yield final inoculum of approximately 5×10^5 CFU/mL. At predetermined time points (0, 2, 4, 6, 8, 10, and 24 hours), a 0.1-mL aliquot was removed from the control tube (drug free) and from the tube with AC or FLC for each strain. Serial dilutions in saline were performed. Volumes of 0.1 mL were spread onto SDA plates and incubated at 35°C for 24 to 48 hours to determine the numbers of CFU/mL. All time-kill curve studies were conducted in triplicate.

Propidium Iodide Staining. To analyze the membrane integrity, fraction of surviving cells of *C. albicans* ATCC 2091 and *C. albicans* n. 4 exposed to AC and each antibiotic were stained with propidium iodide (PI) solution (Sigma-Aldrich). Control samples of both strains were performed for comparison. Briefly, treated and control cells (10^5 cells/mL) were washed and suspended in PBS (pH 7.0). To this cell suspension, PI solution (stock solution 1 mg/mL) was then added to these cell suspensions that were later incubated for 10 minutes at room temperature. Cells were again washed to remove the excess of the stain and examined under the inverted microscope Axio Observer.Z1 with ApoTome.2 (Zeiss, Milan, Italy).

MitoTracker Staining. To detect permeability changes of mitochondrial membrane, fraction of surviving cells of *C. albicans* ATCC 2091 and *C. albicans* n. 4 exposed to AC and each antibiotic were stained with mitochondrion-specific dye MitoTracker RedCMXRos (MTR) (Invitrogen, Fisher Scientific Italia, Rodano-MI, Italy) according to the manufacturer's instructions. Control samples of both strains were performed for comparison. Treated and control cells (10^5 cells/mL) were collected by centrifugation and suspended in fresh medium with the mitochondrion-specific dye MTR at a final concentration of 50 nM.[21,22] Cells were incubated for 15 minutes at 35°C in the dark. Stained cells were washed three times with PBS in the dark and immediately observed using the above-mentioned microscope. MTR has excitation and emission peaks at 579 and 599 nm, respectively.

Ex Vivo Study

Preparation of Inocula. The following strains were used: *C. albicans* ATCC 2091 and *C. albicans* n. 4. The yeasts were cultured in RPMI-1640 medium supplemented with MOPS at 35°C for 24 hours and then, a few colonies of each strain were washed three times with PBS to reach a density of 5×10^8 CFU/mL (V-1200-VWR, Milan, Italy) and then diluted to a final concentration of the inoculum (5×10^4 CFU/mL).

Rabbit Globe Harvest. Normal rabbit eyes, obtained from a local abattoir, were enucleated immediately following

Table 1. MIC Values, μg/mL

Strains	CAF	TET	CS	FLC	AC, Dilutions
C. albicans ATCC 10231	2000	250	>2000	1.25	1:10
C. albicans ATCC 2091	2000	250	>2000	1.25	1:10
C. albicans 4	>2000	500	>2000	1.25	1:20
C. albicans 12	>2000	250	>2000	1.25	1:10
C. albicans 13	>2000	500	>2000	1.25	1:5
C. albicans 15	2000	250	>2000	1.25	1:5
C. albicans 16	>2000	500	>2000	2.5	1:10
C. albicans 18	>2000	500	>2000	2.5	1:10
C. albicans 355	>2000	500	>2000	2.5	1:10
C. glabrata 1	>2000	250	>2000	4	1:5
C. glabrata 3	2000	500	>2000	4	1:20
C. glabrata 8	>2000	500	>2000	4	1:2
C. glabrata 9	>2000	500	>2000	4	1:2
C. glabrata 10	>2000	500	>2000	4	1:5
C. utilis	>2000	500	>2000	2.5	1:2
C. tropicalis	>2000	250	>2000	2.5	1:5

AC: Fixed antibiotic combination containing CAF, TET, and CS, in the fixed ratio 1:1:3.

euthanization, washed before with 1% povidone, rinsed with saline, and after submerged in tubes with 0.1% Dulbecco's Modified Eagle's Medium (DMEM Ham's F-12; PAA Laboratories, Biolife, Milan, Italy). The tubes, placed in a container with ice for preservation, were transported to the laboratory and used immediately.

***C. albicans* Growth Curve.** The growth curves of *C. albicans* ATCC 2091 and *C. albicans* n. 4 ocular isolates were assessed using a modified ex vivo keratitis model.[23]

The eyes were randomly divided into two groups (20 corneas per group) corresponding to each *C. albicans* strain. For each group, the eyes were intrastromally injected with 50 μL of the yeast suspension (5×10^4 CFU/mL) using a 30-gauge needle. Then the groups were divided into five subgroups (four eyes each) according to growth curve times (0, 12, 24, 48, and 72 hours from the insult). The sclero-corneal ring of each eye was excised using curved scissors and placed on the corneal support in dishes containing 2.5 mL DMEM as previously described.[24,25] The organ cultures were incubated at 37°C in humidified atmosphere of 6% CO_2. To moisten the epithelium, 100 μL medium was added drop wise to the surface of the corneal epithelium every 12 hours. The culture medium in the dishes was changed every 24 hours. At established fixed times, corneas without scleral ring were individually weighed and homogenized in 1 mL saline at 4°C for 30 seconds; the homogenate was serially diluted (1:10 dilution) in saline, and seeded in duplicate onto SDA. The results were reported to tissue weight and expressed as CFU/g.

Treatment of *C. albicans* Keratitis. Twenty-four enucleated eyes were randomly divided into two groups (12 corneas per group) corresponding to each *C. albicans* strain. Each group was intrastromally injected with 50 μL yeast suspension containing 5×10^4 CFU/mL. At 2 hours following the injection (early-infection phase), the corneas were divided into three groups of four corneas each. The first group was treated with AC, the second group with FLC, and the third control group with PBS. The treatment regimen consisted of six daily instillations (50 μL). Then the tissues, 1 hour later after the last instillation, were homogenized and plated to determine the number of recovered CFU/g.

Statistical Analysis

The results are expressed as means ± SDs from three experiments and statistically analyzed by a 1-way ANOVA, followed by Tukey posttest by GraphPad Prism Software (San Diego, CA, USA). Differences in groups and treatments were considered significant for $P < 0.05$.

Table 2. The FICI of CAF, TET, and CS Tested in 1:1 Combinations of Each Other Against *C. albicans* Strains

Strains	CAF+TET	TET+CS	CAF+CS
C. albicans ATCC 10231	0.5	0.28	0.12
C. albicans n. 4 (c.s.)	0.5	1	1

FICI: values ≤ 0.5 synergism, values between 0.5 and 1.0 additivity, values > 4.0 antagonism, values between 1.0 and 4.0 indifferent. c.s., clinical strains.

Results

In Vitro Study

Antifungal Susceptibility Testing. MIC_{90} values for the individual antibiotics against all strains of *Candida* spp. used were higher than those observed for the reference FLC. Among all antibiotics tested, TET was the most efficient (MIC: 250–500 μg/mL). MIC values were detected from the 1:2 to 1:20 dilution of the reconstituted AC (Table 1).

Checkerboard. *C. albicans* ATCC 10231 and *C. albicans* n. 4 clinical isolates were chosen for further assays. In a checkerboard microdilution assay, pairs of antibiotics were tested in combination with each other's antibiotic to determine FICI values. Synergy was observed for all combinations tested on *C. albicans* ATCC 10231. In particular, CAF + TET (FICI 0.5), TET + CS (FICI 0.28), and CAF + CS (FICI 0.12). Synergy also was observed for the combination CAF + TET (FICI 0.5) on *C. albicans* n. 4, whereas additivity (FICI 1) was detected for the other combinations (Table 2).

Time-Killing Curve. The time-killing curves showed that the AC and FLC, at a concentration of 10 times MIC, were able to maintain under control the growth of *C. albicans* ATCC 10231 and *C. albicans* n. 4 up to 10 hours. At 24 hours, the AC was able to reduce the loads of 1 Log_{10} CFU/g and 0.8 Log_{10} CFU/g, compared to the initial inoculum for the ATCC strain and for the clinical isolate, respectively (Fig. 1). The differences were statistically significant versus the control, but also versus FLC ($P < 0.0001$ for *C. albicans* ATCC 10231, $P < 0.001$ for *C. albicans* n. 4).

PI Staining. Propidium iodide, a red-fluorescent nuclear stain, is a membrane impermeant dye that is generally excluded from viable cells. Microscopic examination demonstrated that the cells (approximately 86.7%) of both strains treated with AC (1:10 vol/vol) were stained red probably because they lost cell membrane integrity. The cells (approximately 68.3%) treated with CS (1000 μg/mL) demonstrated the same result, whereas 25.6% of untreated cells lost their membrane permeability (Fig. 2). Positively stained cells (PI^+) were observed under inverted fluorescence microscope.

MitoTracker Staining. MitoTracker is a mitochondrion-specific stain in live cells and its accumulation depends on the membrane potential. However, once incorporated in the mitochondria, it can chemically link to thiol groups and will not leave the mitochondria when the membrane potential decreases as a result of fixation and/or cell death. Fluorescence microscope images showed positive staining in mitochondrial networked areas of healthy nontreated cells for both strains. The treated cells (approximately 70%) in the presence of TET (0.5 MIC) or CAF (1000 μg/mL) showed a more diffuse and less intense staining of the mitochondria, indicating that mitochondrial function was reduced but not eliminated. The cells (approximately 90%) treated with AC

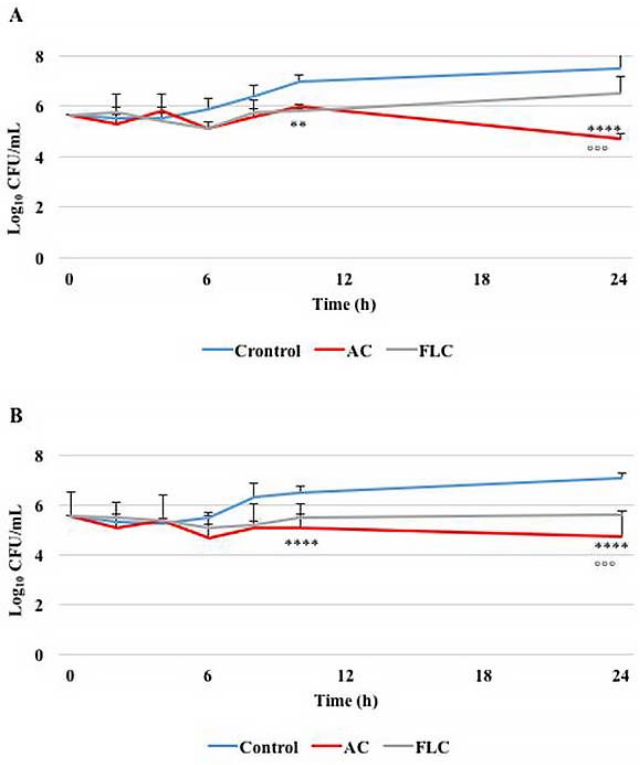

Figure 1. Time-killing curves of *C. albicans* ATCC 10231 (**A**) and *C. albicans* n. 4 clinical isolate (**B**). Mean Log_{10} CFU/mL (± SD) change in *C. albicans* loads treated with AC, FLC, or saline ($^{°°°}P < 0.001$ versus FLC, $^{****}P < 0.0001$ versus control, $^{**}P < 0.01$ versus control).

(1:10 vol/vol) demonstrated similar results. The images showed only a shadow of cells lacking functional mitochondria. Moreover, the staining highlights the morphologic changes of yeast cells treated with AC and antibiotics with respect to the control cells. AC, TET, and CAF effects on mitochondrial function of *C. albicans* ATCC 10231 are shown in Figure 3. The effects on mitochondrial function of *C. albicans* n. 4 was similar to that of *C. albicans* ATCC 10231, therefore not shown.

Ex Vivo Study

***C. albicans* Growth Curve.** The growth curves of *C. albicans* ATCC 10231 and *C. albicans* n. 4 strains were superimposable. The mycotic load obtained from the corneas after intrastromal injection was 3.3 ± 0.5 Log_{10} CFU/g. After 24 hours from the fungal challenge, the load increased approximately 4 Log_{10} CFU/g, remained almost unchanged for up 72 hours, and then decreased (data not shown).

Treatment of *C. albicans* Keratitis. The AC was effective in the ex vivo rabbit keratitis experiments in decreasing the load of *C. albicans* ATCC 10231 and C. *albicans* n. 4. Therefore, AC significantly reduced the load of both *C. albicans* strains by 4 Log_{10} CFU/g with respect to the control after six doses, up to 24 hours after infection ($P < 0.001$). Moreover, AC showed higher activity than FLC against *C. albicans* ATCC 10231 (approximately 1 Log_{10} CFU/g) ($P < 0.01$). Similar efficacy against *C. albicans* n. 4 was observed (Fig. 4).

Discussion

Because fungi are eukaryotic cells, they share many pathways with human cells, thus increasing the probability of antifungal activity of "nonfungal drugs." In the past few years, there has been an increased interest in revived antibiotics. Old drugs that have been recently revived include colistin, temocillin, fosfomycin, mecillinam, nitrofurantoin, and chloramphenicol for multidrug-resistant gram-negative bacteria and trimethoprim-sulfamethoxazole for methicillin-resistant *Staphylococcus aureus*. Among these, colistin and chloramphenicol also demonstrated antifungal activity against yeasts.[14,15,21,26]

In this study, we found that CAF, TET, and CS used alone have weak, if any, antifungal activity against several *Candida* yeasts with respect to FLC, but that this activity is highly enhanced when they were used as AC in fixed combination. The underlying mechanism of each antibiotic against yeasts may be explained as follows: TET and CAF promote mitonuclear protein imbalance and mitochondrial dysfunction, CS binds lipopolysaccharide and anionic phospholipids in the bacterial cell membrane, disrupting membrane integrity.[16,17]

The mechanism of action for tetracycline and its derivative doxycycline is the inhibition of translation through binding to the bacterial 30S ribosomal unit. This specificity for a bacterial

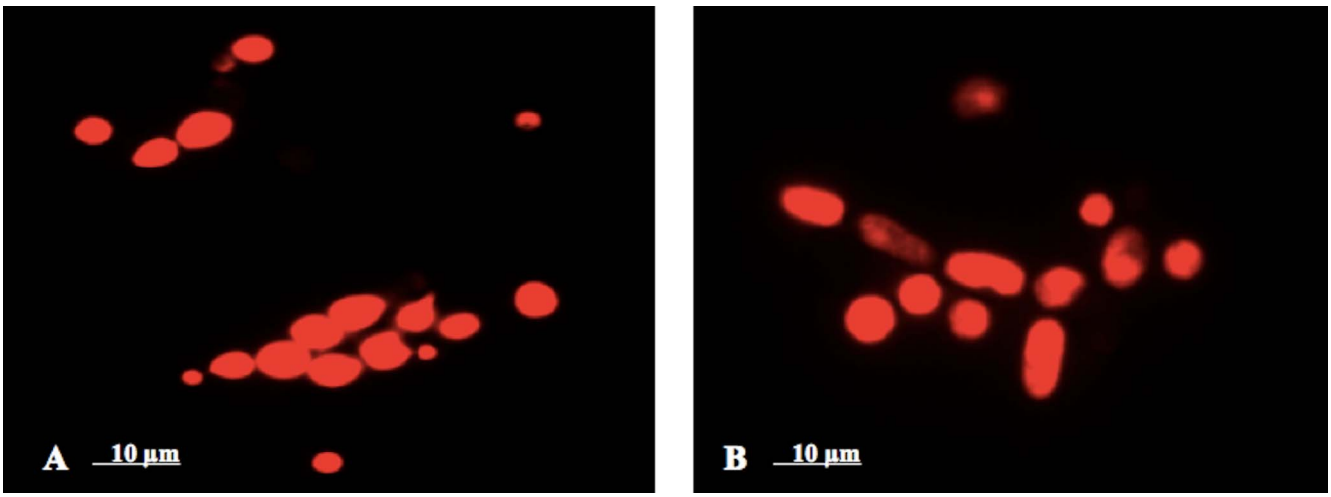

Figure 2. AC and CS effects on membrane permeabilization by PI staining. (**A**) Cells were incubated with CS (1000 µg/mL) for 24 hours before staining. (**B**) Cells were incubated with AC (1:10 vol/vol) for 24 hours before staining. Cells were observed and photographed using inverted fluorescence microscopy.

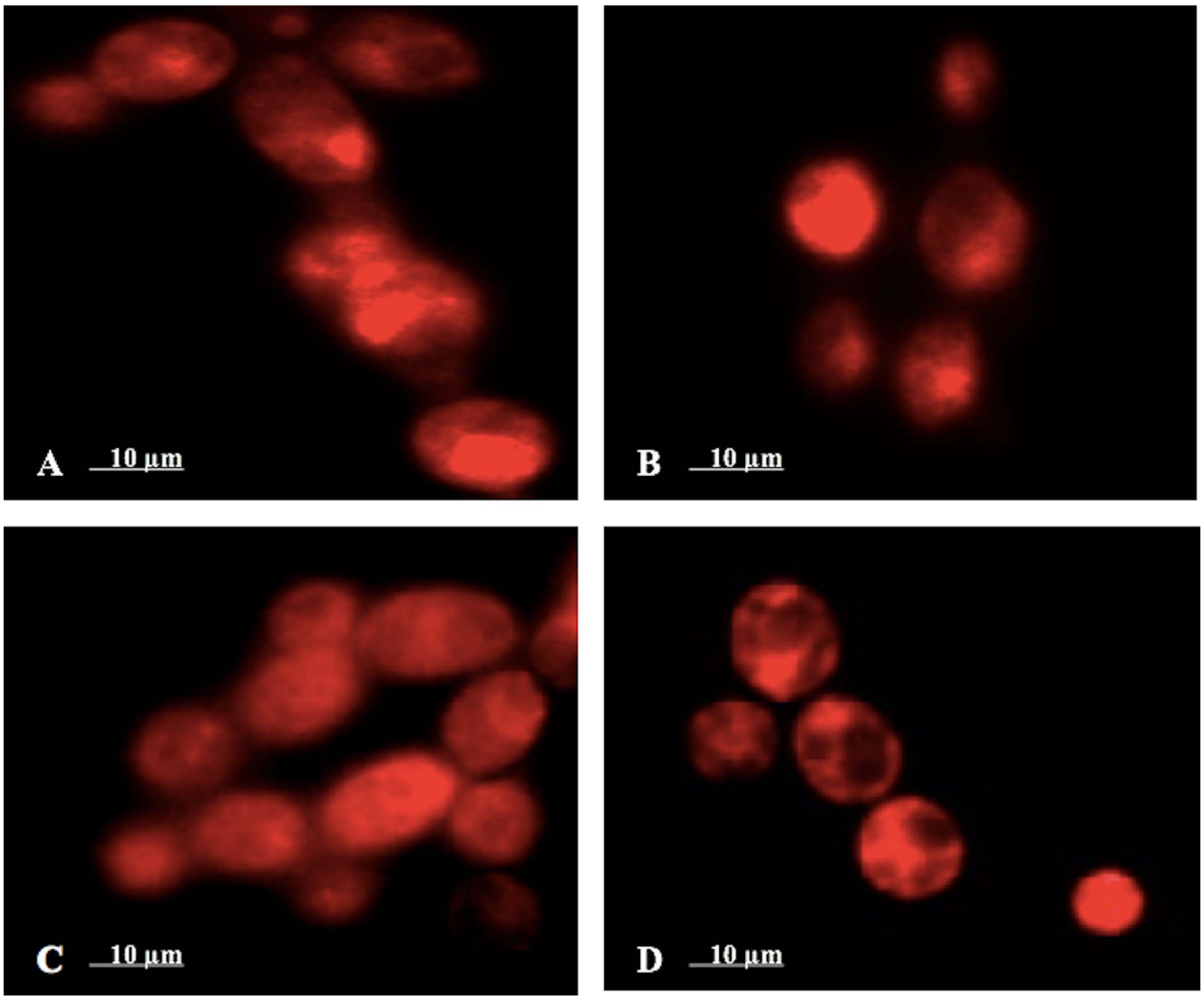

FIGURE 3. AC, TET, and CAF effects on mitochondrial function by MTR staining. (**A**) Cells were incubated with TET at sub-MIC concentration (125 µg/mL) for 24 hours before staining. (**B**) Cells were incubated with CAF (1000 µg/mL) for 24 hours before staining. (**C**) Cells were incubated with AC (1:10 vol/vol) for 24 hours before staining. (**D**) Control, untreated cells at the same time. Cells were observed and photographed using inverted fluorescence microscopy.

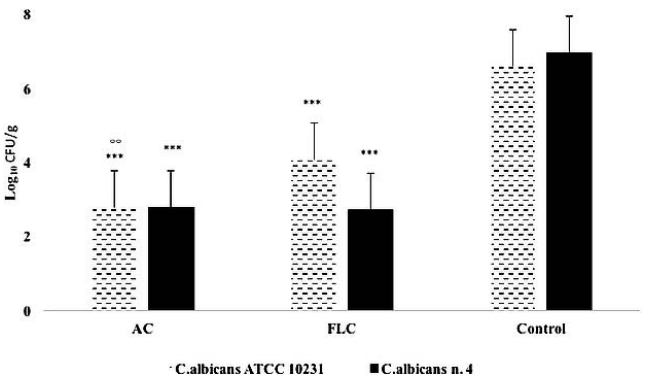

FIGURE 4. Efficacy of AC treatment against *C. albicans* ATCC 10231 or *C. albicans* n. 4 clinical isolate. Mean Log_{10} CFU/g (± SD) change in *C. albicans* ATCC 10231 or *C. albicans* n. 4 loads of AC versus FLC treated or control group in corneal tissue ($^{\circ\circ}P < 0.01$ versus FLC, $^{***}P < 0.001$ versus control).

component has led to an expectation that tetracycline does not affect eukaryotic cells. However, tetracycline leads to a state of so-called mitonuclear protein imbalance, which disturbs mitochondrial proteostasis and inhibits mitochondria function. The mitonuclear protein imbalance is accompanied by a strong decrease in cellular respiration, indicative for severely impaired mitochondrial activity.[16] Moreover, tetracycline eliminates the diauxic shift.[21] The lack of diauxic shift or the lack of a functional mitochondria alters sterol metabolism resulting in lower ergosterol levels.[27]

Chloramphenicol is a known inhibitor of mitochondrial translation in eukaryotes, which binds to the A site and occupies the same position as the aminoacyl-tRNA (aa-tRNA), preventing protein synthesis in prokaryotes.[28] Ribosomal similarities between bacteria and mitochondria may provide the basis for mitochondrial sensitivity to chloramphenicol-mediated inhibition of protein synthesis.[29] Expression of the transferritin receptor seems to be the most relevant to the chloramphenicol-mitochondrion interaction. Specifically, chloramphenicol diminishes mitochondrion-based transferritin

receptor expression, resulting in ferritin depletion in mitochondria.[30]

Polymyxins bind lipopolysaccharide and anionic phospholipids in the gram-negative bacterial cell membrane, disrupting membrane integrity.[17] Polymyxins are cationic cyclic hepatapeptides with a hydrophobic tail that interacts with the bacterial cytoplasmic membrane, therefore changing its permeability and triggering cell death.[26] Weak antifungal activity of colistin and polymyxin B against several fungi has already been reported.[31–33] As hypothesized by Zhai et al.[14] in 2010, polymyxin B kills fungi through binding anionic lipids on the fungal membrane and disruption of membrane integrity. The lower efficiency of polymyxin alone against eukaryotes compared with bacteria could be partly due to the presence of sterols in the eukaryotic membrane, as sterols have been shown to reduce the insertion of cationic peptides into anionic mixed membranes to form pores.

Moreover, these results indicate that the combination of corneal organ culture and experimental microbial keratitis has the potential to be used as a mechanistically based alternative to in vivo animal testing. Although the ex vivo models lack immune elements, the three-dimensional architecture remains, as do the intracellular innate immune molecules and cellular-stromal-components.[34]

In conclusion, we showed that AC containing the three antibiotics in fixed combination has a high efficacy against *Candida* spp., in both in vitro and in ex vivo models. The effect reported can be due to the different modes of action of the three antimicrobial agents used in combination: the CS increases the permeability of the yeast membrane allowing the subsequent penetration of TET and CAF, which alter mitochondrial function.

AC has been widely used on the Italian market and other European countries as an eye drop/ointment to treat bacterial eye infections for 50 years. Based on these results, we can assume that AC has the potential to be used clinically as drug of first choice when the diagnosis of infectious keratitis caused by bacteria or fungi is unclear. Specific diagnostic tests are needed to rule out the condition and, if necessary, progress to appropriate treatment. Further studies are, however, needed on other yeast and mold strains, such as *Fusarium* and *Aspergillus*, to extend these findings.

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