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Evaluation of Synergistic Action Between Nimesulide and the Antifungals Ketoconazole and Nystatin on Clinical Cervical-Vaginal Isolates of *Candida* spp.

Bittencourt F M, Pippi B, Picanço J M A, Alexandre Meneghello Fuentefria A M, Calil L N and Mezzari A.
Department of Analysis, Faculty of Pharmacy, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

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***Corresponding Author Email:** mezzari@ufrgs.br

Abstract
Introduction: Antifungal resistance in cases of vulvovaginal candidiasis (VVC) increasingly affects women worldwide, being a recurrence in gynecological appointments. The need for effective new therapies and the difficulty of introducing antifungals into the pharmaceutical market makes drug combination a quick and interesting therapeutic strategy for solving such a problem. Objective: The present study aims to evaluate the association of nimesulide, a non-steroidal anti-inflammatory drug, with antifungals used in topical treatments for vaginal candidiasis (ketoconazole and nystatin). Secondarily, we link the susceptibility profile of clinical isolates from primary health care units in southern Brazil. Materials and methods: The broth micro dilution methodology was used for susceptibility testing, and the Checkerboard method was used to evaluate the synergistic effect of nimesulide with antifungals. Results and discussions: High percentages of isolates resistant to the tested antifungals were observed: 75% of the isolates were resistant to fluconazole and 36.1% to Itraconazole, as well as 22.2% had low susceptibility to ketoconazole and 55% to nystatin. The combination of nimesulide and ketoconazole showed synergistic effect for 62.5% of *Candida* spp. tested, including *C. albicans*, *C. glabrata* and *C. tropicalis* isolates. On the other hand, the combination of nimesulide and nystatin resulted in 100% indifference.

Keywords
Antifungal resistance, Synergism, Nimesulide, Ketoconazole, Nystatin, Candidiasis.

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INTRODUCTION
The vulvovaginal candidiasis (VVC) is an opportunistic infection cause by the uncontrolled growth of *Candida* spp. yeasts in the female genital tract, being favored in immunocompromised individuals and when there are alterations in the vaginal microbiota (1,25). The VVC affects millions of women and is the most common cause of acute vaginitis in Europe, United States and some tropical countries (21). It is estimated that approximately 75% of women in fertile age are affected throughout life and 5% of those present recurrent vulvovaginal candidiasis (RVVC), with 4 (four) or more symptomatic episodes in a year (27).

Studies show that *C. albicans* is the most frequent species, being responsible for 50-60% of the VVC (5,15). However, the non-*albicans* species are emerging, especially in recurrent episodes, developing a worrisome situation due to the high resistance of these microorganisms to antifungals (4,6,19). The subjectivity of the symptoms and the great discomfort caused to the patients lead to uncontrolled use of antifungals without proper diagnosis; this indiscriminate use contributes to the emergence of microorganism's resistant to antifungal therapy (17,23).

Due to the growing isolation of resistant strains and the difficulty of introducing new antifungals in the pharmaceutical market, the combination of drugs becomes an interesting therapeutic strategy (14). The use of nonsteroidal anti-inflammatory drugs (NSAIDs), like nimesulide, is already being used by some doctors to ease the symptoms of candidiasis (10,24). The nimesulide is a selective inhibitor of the enzymes that synthesize the prostaglandins, the cyclooxygenases (COX), specially COX-2. In addition to this main mechanism of action, it has other biochemical properties responsible for the therapeutic action. Among them, the inhibition of toxic oxygen radicals, decreased enzyme secretion, as well as the decreased histamine release (2).

Based on such information, the present study seeks to analyze the antifungal action of fluconazole, litraconazole, ketoconazole, nystatin and nimezulide on Candida spp. isolates of cervicovaginal origins. Furthermore, with the objective of showing the use of anti-inflammatory drugs as a complementary treatment to the antifungal therapy, we evaluated the antifungal action of the association of nimesulide with ketoconazole and nystatin.

MATERIALS AND METHODS

Fungal Isolates:

A total of 36 clinical isolates of *Candida* spp. were included in this study. The isolates come from cervical-vaginal collections and are stored at the Fungal Library of the Department of Analysis of the Faculty of Pharmacy of the Federal University of Rio Grande do Sul, Porto Alegre, RS. The species were identified previously by the micro culture in slide technique and through seeding in chromogenic agar (CHROMagar™, Candida - Difco). Before the experiments, all the isolates were sub cultured on Sabouraud dextrose agar with chloramphenicol (HIMEDIA) and incubated at 35°C for 24 hours.

Antifungals and Anti-inflammatories:

The stock solutions of antifungals were prepared as recommended by the protocol M27-A3 of the *Clinical Laboratory Standards Institute* (8). Stock solutions of fluconazole were prepared in distilled water; and the stock solutions of itraconazole, ketoconazole and nystatin, as well as the stock solution of the anti-inflammatory nimezulide, were prepared in dimethyl sulfoxide (DMSO; Nuclear, Brazil). For the tests, the drugs were diluted in RPMI 1640 medium (Roswell Park Memorial Institute 1640; Sigma-Aldrich, USA) buffered with MOPS (morpholinopropanesulfonic acid; Sigma-Aldrich) and adjusted to pH 7, in order to obtain a maximum concentration of 1% of DMSO.

Susceptibility Test:

The susceptibility tests to antifungals from the 36 isolates were performed through the method of micro dilution in broth, accordingly to the technical standards published in the document M27-A3 of the CLSI (8). Serial dilutions were performed with RPMI 1640 medium (Sigma-Aldrich, USA), obtaining the following ranges of test concentrations: 64 – 0,125 µg/mL (fluconazole e nystatin), 16 – 0,0312 µg/mL (ketoconazole and itraconazole) e 256 – 0,5 µg/mL (nimezulide). The minimum inhibitory concentrations (CIM) were defined as the smallest concentrations in which could not be observed visible growth (nystatin) or could be observed decrease of 50% (azoles and nimezulide) of growth in 48 hours. The criteria of definition of susceptibility to the fluconazole and itraconazole were defined according to CLSI M27-A3 considering the updates published in the document M27-S4 (8,9). For the ketoconazole and nystatin, criteria of susceptibility were stablished according to previous studies (11,16).

Evaluation of the association of nimezulide with antifungals by Checkerboard assay:

The interaction of nimezulide with ketoconazole and nystatin was analyzed in 8 isolates from *Candida* spp. through the checkboard method (18). The assay allowed to evaluate 49 different combinations between the anti-inflammatory and the antifungal agents. The trials were performed in duplicate and incubated at 35°C for 48 hours. The effect of the combinations was assorted by the determination of the fractional inhibitory concentration index (FICI), expressed as the sum of the fractional inhibitory concentrations (FIC), as defined by the following equation:

$FICI = FIC_A + FIC_B = (MIC_A \text{ combined}) / (MIC_A \text{ alone}) + (MIC_B \text{ combined}) / (MIC_B \text{ alone})$

where MIC_A and MIC_B are the MICs of the antifungal agent and the anti-inflammatory, respectively (Murkherjee et al, 2005). Synergism was defined when $FICI \leq 0.5$, indifference when $0.5 < FICI \leq 4$ and antagonism when $FICI > 4$ (18).

RESULTS AND DISCUTION

The most frequent species of *Candida* isolated from vaginal collections is *C. albicans*, corresponding to approximately 55% of the cases with clinical symptoms (5,15). However, the emergence of non-*albicans* species and the relation between these species with higher incidence of resistance to antifungals are changing the etiology of candidiasis (19). Of the 36 samples of *Candida* spp. evaluated in this study, 36.1% were *C. albicans*, corresponding to a smaller percentage compared to epidemiological studies described in the literature (5,15,19,27). From the non-*albicans* species, the most frequent was *C. glabrata* (27.7%), followed by *C. krusei* (16.6%), *C. tropicalis* (8.3%), *C. parapsilosis* (8.3%) and *C. guilliermondii* (2.7%).

These isolates presented variable susceptibility to antifungals. The MICs varied from 1 to > 64 µg/mL to flucanozole, from 0,03 to > 16 µg/mL to ketoconazole, from 0,25 to > 16 µg/mL to itraconazole and from 2 to 8 µg/mL to nystatin. Regarding nimezulide, no isolate was sensible to this anti-inflammatory (MIC > 256 µg/mL). The results of the susceptibility test for all the evaluated drugs are demonstrated in Table 1.

Table 1: Values of minimal inhibitory concentrations (MIC) from fluconazole (FLC), ketoconazole (KEC), itraconazole (ITC) and nimezulide (NMZ) against the isolates of Candida spp.

SPECIES	ISOLATE	MIC (µg/mL)				
		FLC	ITC	KEC	NYT	NMZ
C. albicans (n=13)	CA06	16*	0,5	16*	4*	>256
	CA07	>64*	0,5	16*	8*	>256
	CA09	32*	0,5	0,125	2	>256
	CA10	16*	0,5	0,125	2	>256
	CA11	16*	1*	NR	4*	>256
	CA12	16*	0,5	NR	4*	>256
	CA13	32*	1*	NR	4*	>256
	CA14	16*	1*	0,03	4*	>256
	CA15	8*	>16*	>16*	4*	>256
	CA16	4	>16*	>16*	2	>256
	CA17	8*	>16*	>16*	2	>256
	CA18	>64*	>16*	>16*	4*	>256
	CA19	8*	0,5	4	2	>256
C. glabrata (n=10)	CG02	>64*	0,5	2	2	>256
	CG04	16	1*	2	2	>256
	CG07	16	0,5	0,25	4*	>256
	CG08	1	0,25	0,5	2	>256
	CG09	>64*	>16*	4	2	>256

	CG13	2	0,5	0,125	2	>256
	CG15	2	0,5	0,125	2	>256
	CG17	>64*	1*	8	2	>256
	CG18	>64*	1*	32*	2	>256
	CG20	0,5	0,25	0,03	2	>256
C. guilliermondii (n = 1)	CGUI01	8	0,5	1	2	>256
	CK01	>64*	0,5	1	4*	>256
	CK02	>64*	0,25	0,5	2	>256
	CK03	16*	0,25	0,03	4*	>256
C. krusei (n=6)	CK04	>64*	0,5	1	8*	>256
	CK06	8*	0,5	0,06	4*	>256
	CK12	16*	0,5	0,03	4*	>256
	CP01	4	0,125	0,03	4*	>256
C. parapsilosis (n=3)	CP04	>64*	0,25	0,03	4*	>256
	CP11	8*	0,25	0,06	4*	>256
	CT01	>64*	4*	>16*	4*	>256
C. tropicalis (n=3)	CT03	16*	0,5	0,25	4*	>256
	CT04	>64*	1*	0,25	4*	>256

NR = Not realized; *Resistant or strains with low susceptibility

It could be observed that 75% of the isolates were resistant to fluconazole and 25% presented dose-dependent sensibility. None of the isolates were sensible to this antifungal. Related to itraconazole and ketoconazole, 36.1% and 22.2% of the isolates were resistant, respectively. Also, approximately 55% of the *Candida* spp. isolates had low susceptibility to nystatin, a polyene antifungal. Santos et al. (2005) (23) found broad sensibility to fluconazole of *C. albicans*, and the non-*albicans* species were completely resistant in patients with recurrent vulvovaginal candidiasis. [10] Studies performed by Feng et al. (2018) showed that 38.7% of the *Candida* spp. isolates were resistant to itraconazole (12). However, in a study performed by Pérez et al. (2016), all the tested *Candida* isolates were susceptible to nystatin (22).

Currently, the treatment for vulvovaginal candidiasis lean on the use of nystatin and oral or topic azole derivatives. In cases of recurrent candidiasis, a suppression regimen with prolonged doses of oral antifungal or any topical azole for seven to 14 days, such as ketoconazole, is initially required (13). However, as showed in this study, resistant clinical isolates are frequently found. The most immediate solution to avoid such problem of resistance may be the association between drugs to potentialize the clinical action (7). The investigation of the efficiency of NSAIDs combined with antifungals has showed great value in the attempt to eradicate the microbial resistance (28,29). To investigate the synergic effect of nimezulide with ketoconazole and nystatin, isolates that presented resistance or low susceptibility to the tested antifungals were selected. The MICs of the combination, the FICI and

the type of interaction obtained in the checkerboard assay are shown in Table 2.

Table 2: In vitro susceptibility of Candida spp. to nimezulide (NMZ) combined with ketoconazole (KEC) and nystatin (NYT).

ISOLATE	NMZ + KEC				NMZ + NYT			
	MIC of the combination (µg/mL)				MIC of the combination (µg/mL)			
	NMZ	KEC	FICI	Interaction	NMZ	NYT	FICI	Interaction
C. albicans CA07	4	2	0,13	SYN	256	2	0,75	IND
C. albicans CA18	32	2	0,09	SYN	4	4	1	IND
C. glabrata CG09	32	1	0,31	SYN	4	2	1	IND
C. glabrata CG18	4	2	0,07	SYN	4	4	1	IND
C. krusei CK01	256	0,25	1	IND	4	8	1	IND
C. krusei CK04	256	0,5	1	IND	4	8	1	IND
C tropicalis CT01	16	0,5	0,06	SYN	4	4	1	IND
C tropicalis CT04	4	0,25	1	IND	4	4	1	IND

FICI: fractional inhibitory concentration index; SYN: synergism; IND: indifference

Through data analysis we can observe that the association of ketoconazole with nimezulide resulted predominantly in synergic interaction, with this effect being observed in 62.5% of the *Candida* spp. isolates. The association obtained relevant results for the two tested isolates of *C. glabrata* and *C. albicans*, as well as for one of the isolates from *C. tropicalis*. The *C. krusei* isolates did not shown higher susceptibility against the combination. On the other hand, nystatin combined to nimezulide resulted in 100% indifference. Antagonism was not detected in both combinations. Such results corroborate with studies performed by Yücesoy et al. (2000), in which the combination of fluconazole with other NSAIDs, such as sodium salicylate, tenoxicam and diclofenac sodium, has shown synergic activity (29). Costa-de-oliveira et al. (2015) (10) and Sharma et al. (2015) (24) had also stated that the association of the NSAID ibuprofen with fluconazole is a good therapeutic alternative to the reversion of resistance in *Candida* spp. Species (10,24).

During the infection, the presence of fungal antigens leads to the synthesis of prostaglandins, whose cascade is activated by the COX, which are responsible to the inflammatory mechanism (26). The *Candida* genus is capable of producing enzymes similar to the mammalian COX, which interfere in the fungal metabolism, raise the transition of yeasts to the hyphal state and contribute to virulence (20). Therefore, the concomitant administration of COX inhibitors, such as NSAIDs, with antifungals can be an efficient alternative in the therapy for candidiasis. Thus, the use of nimezulide could have potentialize the action of ketoconazole by blocking the fungal growth and preventing the transition to the hyphal state, an important factor to the installation of the infection. Also, there are anti-inflammatory advantages of this drug related to the clinical manifestations of the disease and, therefore, better *in vivo* results can be achieved (3,28).

CONCLUSION

The data obtained, as well as the bibliographic survey realized, suggest that NSAIDs have an important and promising role in the control of fungal infections caused by species of *Candida* spp. In the present study, the results demonstrated that the combination of nimezulide with ketoconazole can be useful to the treatment of vulvovaginal candidiasis, especially for the ones caused by strains resistant to azoles. However, additional pre-clinical trials and *in vivo* studies are necessary to define its clinical use.

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