

Sensitivity of mixed cultures of microorganisms to the new combined drug in the form of pessaries

I. V. Nizhenkovska, L. V. Onyshchuk* and O. I. Nizhenkovskiyi

Department of Pharmaceutical, Biological and Toxicological Chemistry,
Bogomolets National Medical University, Ukraine.

ABSTRACT

The inhibitory properties of clotrimazole, metronidazole, and ibuprofen against biofilms of both bacteria and fungi were studied. The distinct antibiofilm effect of a potential combined drug MCI-4 against *Staphylococcus aureus*, yeast-like fungi, and microbial associations formed by *Staphylococcus aureus* 222 and *Candida albicans* 1486 was experimentally proven.

KEYWORDS: vaginosis, biofilms, *Staphylococcus aureus*, *Candida albicans*, metronidazole, clotrimazole, ibuprofen, pessaries.

INTRODUCTION

Infectious inflammatory diseases of the female genital organs are a serious medical and social problem in obstetrics and gynecology [1, 2].

Successful treatment of these diseases depends on the correct and timely diagnosis and appropriate pathogenetic therapy. The goal of treating vaginitis and vaginosis is to inhibit pathogenic microorganisms, inhibit the inflammatory process, and restore normal vaginal microflora [3].

According to modern concepts, the main form of existence of microorganisms is biofilms - structurally organized microbial communities attached to an abiotic (implants, catheters) or biotic (macroorganism tissue) substrate and surrounded by a biopolymer matrix. Bacterial

cells in biofilms are characterized by a high level of pathogenicity, resistance to the protective mechanisms of the human body, and resistance to antibiotics and disinfectants [4, 5]. Biofilms reduce the effectiveness of antimicrobial chemotherapy and contribute to the chronization of the inflammatory process; they are detected in 80-90% of cases of chronic diseases [6, 7].

A new alternative and effective drug for intravaginal use was created in an early study based on our previous research conducted at the Department of Pharmaceutical Technology and Biopharmacy Department, Shupyk National Medical Academy of Postgraduate Education, Ukraine under the direction of Professor L. L. Davtyan for the treatment of female genital diseases, the effectiveness of which is the result of three components: Ibuprofen, Metronidazole and Clotrimazole [8].

The study was conducted to investigate the effect of a potential combination drug MCI-4 with antimicrobial and anti-inflammatory activity in the form of pessaries on the sensitivity of biofilms of *Staphylococcus aureus*, *Candida albicans* and their microbial associations.

MATERIALS AND METHODS

Within the study the samples of a combined drug were used in the form of pessaries, which includes clotrimazole, metronidazole, ibuprofen, and witepsol.

Experiments were conducted using a clinical strain of *Staphylococcus aureus* (*S. aureus*) 222,

*Corresponding author: lucy.on04@gmail.com

sensitive to azithromycin, amikacin and clindamycin, moderately sensitive to erythromycin, and resistant to oxacillin, gentamicin, rifampicin and ciprofloxacin and *Candida albicans* (*C. albicans*) 1486, sensitive to clotrimazole, itraconazole and terbinafine, moderately sensitive to amphotericin B, and resistant to fluconazole and ketoconazole.

Antibacterial effect of the new combined drug in relation to the planktonic form of *S. aureus* was determined by serial dilutions and evaluated by minimum inhibitory concentration (MIC) indicators [9]. Experiments were conducted using Muller-Hinton broth.

The ability of the studied substances to influence the film formation of bacteria and fungi was studied on 1-day cultures of microorganisms using enzyme-linked immunosorbent assay (ELISA). Optical density measurements were performed on an absorption Microplate Reader ELx800 spectrophotometer. The control medium was microbial cultures grown under the same conditions, without adding compounds and preparations [10].

The antimicrobial effect of pessaries was studied according to the State Pharmacopoeia of Ukraine [11]. The final concentration of clotrimazole was 5000 mcg/ml, 500 mcg/ml and 50 mcg/ml, metronidazole - 1250 mcg/ml, 125 mcg/ml and 12.5 mcg/ml, and ibuprofen - 1000 mcg/ml, 100 mcg/ml and 10 mcg/ml.

Statistical processing of research results was performed using Excel 2007 programs (Microsoft Corp., USA) and Statistica 6.0 (StatSoft, USA). The variance method (analysis of variance (ANOVA)) and the Newman-Keiles criterion were used to evaluate research results and identify differences [12].

RESULTS AND DISCUSSION

The effect of the experimental MCI-4 sample on film formation was studied in relation to aerobic (*S. aureus* 222) and anaerobic (*C. sporogenes* ATCC 19404) bacteria and yeast-like fungi (*C. albicans* 1486).

At the first stage, the sensitivity of planktonic cells of bacteria and fungi to the active ingredients of MCI-4 pessaries clotrimazole and metronidazole was determined, with the establishment of minimum inhibitory concentration (MIC). The experiments used the clotrimazole and metronidazole substances.

The results of the experiments showed (Table 1) that MIC of clotrimazole is 1.95 mcg/ml in relation to *S. aureus* 222 and 1.56 mcg/ml in relation to *C. albicans* 1486, while MIC of metronidazole is 0.015 mcg/ml in relation to *C. sporogenes* ATCC 19404, which indicates their sensitivity to the action of the studied substances [13].

Since the film formation of microorganisms depends on their adhesive properties, the ability of test strains of bacteria and fungi to adhere to the abiotic surface was determined at the next stage of research. The data obtained are shown in Table 2.

The results show that according to reference [14], *S. aureus* 222 is characterized by an expressive ability to adhere, *C. albicans* 1486 – average; in *C. sporogenes* ATCC 19404 adhesion ability was not detected. Due to the lack of adhesive properties in *C. sporogenes*, this microorganism (as a monoculture) was not used in further experiments.

When studying the antibiofilm activity of clotrimazole and metronidazole, the concentrations of these substances in pessaries (clotrimazole – 400 mg,

Table 1. Antimicrobial effect of individual active substances of MCI-4 pessaries.

Microorganisms	MIC, mcg/ml	
	Clotrimazole	Metronidazole
<i>S. aureus</i> 222	1.95	-
<i>C. albicans</i> 1486	1.56	-
<i>C. sporogenes</i> ATCC 19404	-	0.015

Note: «-» – no studies were conducted, because the microorganism is not sensitive to the substances.

Table 2. Adhesive properties of test strains of microorganisms.

Microorganisms	OD value	Film formation ability
<i>S. aureus</i> 222	0.460	Strong
<i>C. albicans</i> 1486	0.103	Average
<i>C. sporogenes</i> ATCC 19404	0.048	Absent

metronidazole – 100 mg) were taken into account. In experiments to determine the film formation under the effect of active ingredients of pessaries, clotrimazole concentrations were 100 mcg/ml, 200 mcg/ml, 400 mcg/ml, and metronidazole – 50 mcg/ml, 100 mcg/ml, 200 mcg/ml. The results obtained are presented in correspondingly published studies [7] and showed that clotrimazole in the studied concentrations (100-400 mcg/ml) exhibits distinct activity against biofilms of *C. albicans* and inhibits the film formation by 92.2-93.7%. The data indicated a clear dose-dependent antibiofilm effect of metronidazole against *S. aureus*: the maximum inhibitory activity is observed at a concentration of 200 mcg/ml (75.1%), the minimum – at 50 mcg/ml (43.3%).

The antibiofilm activity of metronidazole against yeast-like fungi is observed only at a concentration of 200 mcg/ml, while the inhibitory effect is 57.3%. A decrease in the concentration of metronidazole to 50 mcg/ml is accompanied by a decrease in the antibiofilm activity of metronidazole, as evidenced by the accumulation of *C. albicans* 1486 biomass.

The components of pessaries, in addition to antimicrobial substances, also include the nonsteroidal anti-inflammatory drug – ibuprofen, which, according to references [15, 16], exhibits antimicrobial effect, in particular antibiofilm effect, and can also enhance the specific activity of antimicrobial drugs. The study data show that ibuprofen has a distinct antibiofilm effect against *S. aureus*. Thus, at a concentration of 20 mcg/ml, the inhibitory effect is 50.8%, and at 40 mcg/ml and 80 mcg/ml – 76.7% and 72.3%, respectively.

Thus, the conducted experiments showed a clear effect of the active substances of pessaries against monocultures of planktonic aerobic gram-positive and obligate anaerobic bacteria and yeast-like fungi, and their effect against biofilms formed by

a monoculture of bacteria or fungi was also proved.

Ibuprofen has been found to have a distinct inhibitory effect on biofilms of *C. albicans* 1486: inhibition in the range of 63.4-70.0%, depending on the concentration.

It is known that biofilms in most cases are polymicrobial, represented by different types of microorganisms, and hence at the next stage of research it was advisable to determine the antibiofilm activity against mixed cultures. Various microbial associations were used in the experiments: (*S. aureus* 222 + *C. albicans* 1486); (*S. aureus* 222 + *C. sporogenes* ATCC 19404); (*C. albicans* 1486 + *C. sporogenes* ATCC 19404); (*S. aureus* 222 + *C. albicans* 1486 + *C. sporogenes* ATCC 19404). Since mixed cultures contain microorganisms with different oxygen requirements, experiments were performed under aerobic and anaerobic conditions. The results of the experiments made it possible to select a mixed culture (*S. aureus* 222 + *C. albicans* 1486), which has the most expressed ability to form films. It should be noted that under aerobic conditions, the adhesive properties of the association (*C. albicans* 1486 and *C. sporogenes* ATCC 19404) are amplified.

Since the combined use of several antimicrobial agents can both increase the activity and level the action of individual components, the next stage of research was to determine the antibiofilm effect of the active ingredients of pessaries by their combined effect on test microorganisms. Experiments were conducted on monocultures (*S. aureus*, *C. albicans*) and microbial associations (*S. aureus* 222 + *C. albicans* 1486). The concentration of the studied substances was: clotrimazole – 400 mcg/ml, metronidazole – 100 mcg/ml, ibuprofen – 80 mcg/ml. The data obtained are shown in Figure 1.

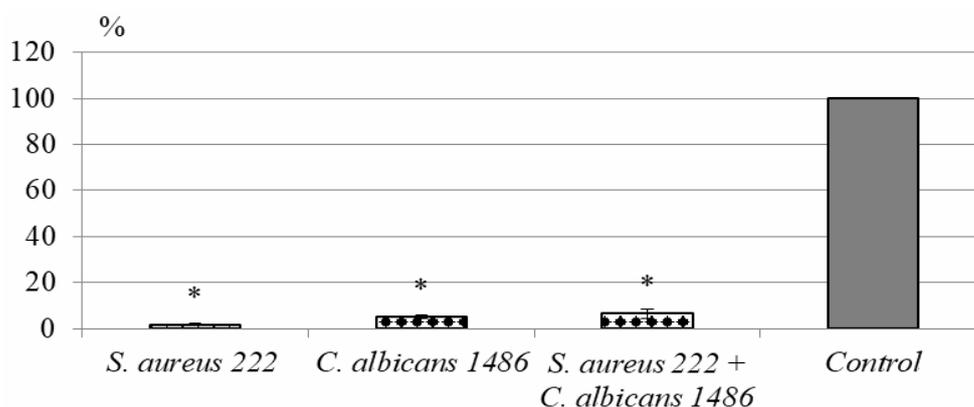


Figure 1. Film formation of bacteria and fungi with the combined effect of clotrimazole, metronidazole and ibuprofen (% of the biofilm formed). Note: * - differences are likely compared to the control ($P < 0.05$).

Results of the conducted studies (Figure 1) indicate that the active substances of pessaries, when used together, show distinct effect – biofilms of bacteria and fungi are practically not formed. It was found that the inhibitory effect on *S. aureus* 222 biofilm is 98.3%, *C. albicans* biofilm – 94.6%, *S. aureus* 222 + *C. albicans* 1486 biofilm – 93.5% ($P < 0.05$ compared to the control).

In further experiments, the film formation of bacteria, fungi, and microbial associations under the effect of pessaries was determined. The results of the experiments showed that the test pessary sample under study dose-dependently suppressed film formation of *S. aureus* 222; the inhibitory effect was 12.0-93.5%, depending on the concentration of active substances.

Clear effect of pessaries was also found in relation to biofilms formed by yeast-like fungi; inhibition of film formation was 82.4-94.8%, depending on the concentration of active components of pessaries.

Results of studies on the formation of biofilms by mixed culture (*S. aureus* 222 + *C. albicans* 1486) under the effect of test pessaries are shown in Figure 2. The results of the experiments showed that MCI-4 inhibited film formation by the mixed culture in a dose-dependent manner; with the maximum studied concentration of clotrimazole (5000 mcg/ml, dilution 1:80), the inhibition was 81.7%. With a decrease in the concentration by 10 and 100 times, a decrease in the antibiofilm effect was recorded; the inhibitory activity was 29.6% and 18.0%, respectively.

Since excipients, in particular the base of pessaries, can have a negative effect on the specific antimicrobial effect of active substances, we investigated the effect of witepsol on the formation of biofilms by microorganisms (Figure 3). The content of witepsol in pessaries was up to 4000 mg.

The data obtained showed that witepsol prevented film formation by yeast-like fungus *C. albicans* 1486 (inhibition of 82.2%), did not affect the formation of biofilm by *S. aureus* and contributed to a 2.5-fold increase in mixed culture biomass compared to the control medium.

CONCLUSION

The results of the conducted studies showed that the active substances of pessaries when they were used together showed expressed effect - biofilms of bacteria and fungi almost did not form. It was found that the inhibitory effect on *S. aureus* biofilm was 98.3%, *C. albicans* biofilm – 94.6%, and *S. aureus* + *C. albicans* biofilm – 93.5% ($P < 0.05$ compared to the control). The data obtained showed that witepsol (the base of pessaries) prevented film formation by yeast-like fungus *C. albicans* (inhibition of 82.2%), did not affect the formation of biofilm by *S. aureus* and contributed to a 2.5-fold increase in mixed culture biomass compared to the control.

The results of the experiments showed that MCI-4 suppressed film formation by mixed culture dose-dependently, while inhibition at the maximum

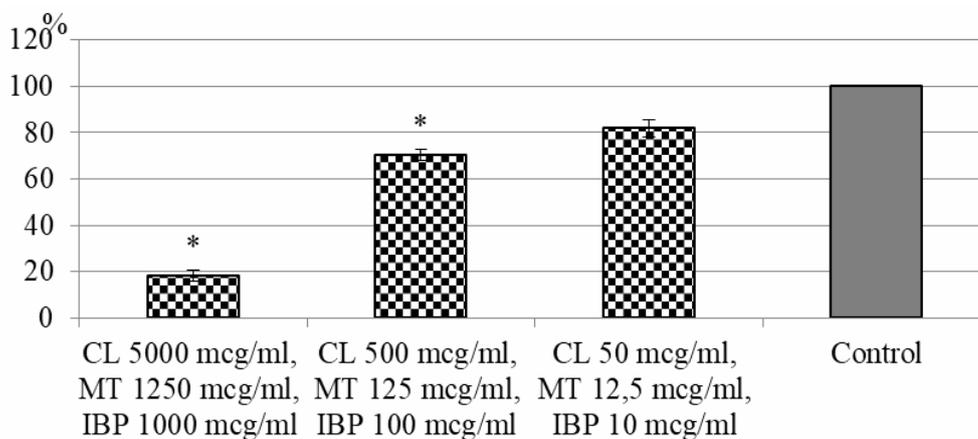


Figure 2. Effect of MCI-4 pessaries on film formation of mixed culture (*S. aureus* 222 + *C. albicans* 1486) (% of the biofilm formed). Notes: CL - clotrimazole; MT - metronidazole; IBP - ibuprofen; * - differences are likely compared to the control ($p < 0.05$).

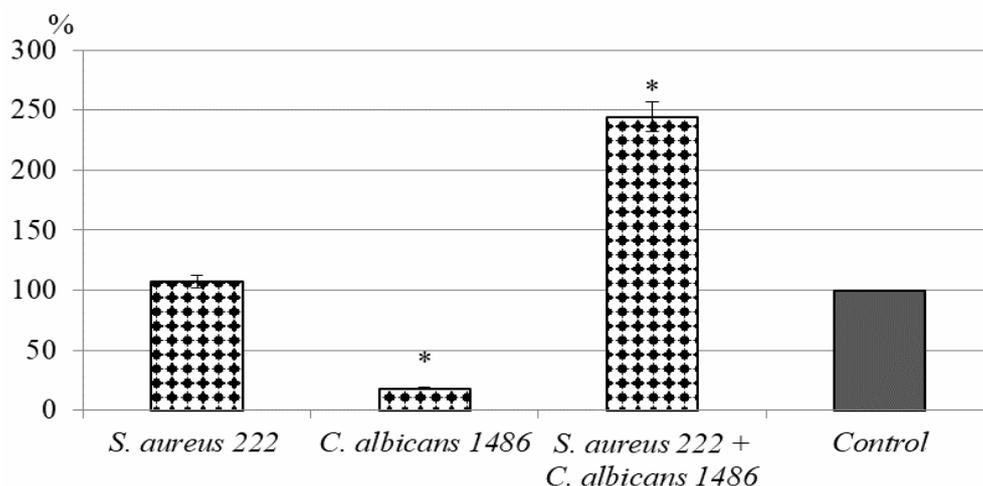


Figure 3. Effect of witepsol on the formation biofilms of *S. aureus* 222, *C. albicans* 1486 and microbial associations. Note: * - differences are likely compared to the control ($P < 0.05$).

studied concentration of clotrimazole (5000 mcg/ml, dilution 1:80) was 81.7%. With a decrease in the concentration by 10 and 100 times, a decrease in the antibiotic film effect was recorded; the inhibitory activity was 29.6% and 18.0%, respectively.

It should be noted that the antimicrobial effect of MCI-4 is realized not only in relation to planktonic microorganisms, but also in relation to biofilms. These data indicate the prospects and feasibility of using MCI-4 in acute and chronic inflammatory processes caused by biofilms.

CONFLICT OF INTEREST STATEMENT

We assert that there are no conflicts of interest.

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