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# SYNERGISTIC ANTIMICROBIAL ACTIVITY BY COMBINING TERBINAFINE WITH BENZOYL PEROXIDE AGAINST CANDIDA ALBICANS AND STAPHYLOCOCCUS AUREUS

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***The aim.*** *To investigate the ability of microorganisms' C. albicans and S. aureus to form biofilms. To evaluate the sensitivity of biofilms to combination of terbinafine and benzoyl peroxide on this association.*

***Material and methods.*** *The ability to form biofilms of microorganisms and the antimicrobial effect of the studied drugs was carried out on polystyrene plates for enzyme-linked immunosorbent assay. The optical density (OD) of biofilms was measured at a wavelength of 545 nm on a biochemical analyzer. The viability of microorganisms was determined by counting the number of colony-forming units (CFU) in 1 ml of the culture medium with experimental strains.*

***Results.*** *The average optical density of biofilms was (1.0892±0.006) units. The OD for clinical isolates was (0.0776±0.004) units. It has been proven that combination of the antimycotic substance − terbinafine and the antiseptic − benzoyl peroxide had a high activity in relation to the association of C. albicans and S. aureus with concentration of 1.3 μg/ml.*

***Conclusion.*** *The study showed the ability to form biofilms in clinical strains of microorganisms is more pronounced than the reference strains. The highest rate of biofilm formation was found in the association of microorganisms' C. albicans and S. aureus. The combination of medicines also effectively operated with planktonic forms of bacteria, but also on microorganisms mobilized in biofilms. A decrease of optical density and 2-fold decrease of CFU demonstrated this.*

***Key words:*** *polymicrobial infection, biofilms, combined action.*

The interest of researchers about interaction of microorganisms in the association increased in the recent decades. They actively interact with each other and form biofilms. There are they specifically organized and have ability to attach the surface [1]. The biofilm of microorganisms is more resistant to the action of disinfectants, antibacterial drugs, bacteriophages, antibodies and phagocytes [2, 3]. It became more problematic to fight these infections considering these factors. An actual task of modern medicine is searching of new effective drugs that will acceptable to inhibit the formation of biofilms is [4, 5].

It has been proven that *Candida albicans* and *Staphylococcus aureus* are capable to form polymicrobial biofilm. In this form of microorganisms they increase their pathogenic potential for several times [6, 7]. Infections caused by this association lead to the death in developed countries. According to the literature, there are 27% of cases the association of these microorganisms caused of nosocomial infections and in 11% − catheter-associated infections [8]. Australian scientists have found that staphylococcal-candidiasis infection contributed acute postpartum mastitis in women in 20% [9].

Microorganisms *C. albicans* and *S. aureus* colonize the mucous membranes of the oral cavity, skin and vagina of a healthy person. At the same time, these pathogens can cause severe diseases of the upper respiratory tract, skin, genitourinary system, stomatitis, burn wounds, etc. adhere to both damaged and undamaged native surfaces [10, 11]. Such infections occur as a chronic course. The majority of these infections occur in persons with multiple risk factors for infection. More detailed discussions of the clinical manifestations of staphylococcal and candida diseases can be found in several recent reports. The majority of these infections occur in persons with multiple risk factors for infection. The use of antimicrobial therapy often becomes ineffective in these cases [12].

A scientist conducted an experiment to determine the mechanisms of interaction microorganisms in associations. They studied patterns of formation of heteromicrobial biofilms, factors of their pathogenicity and virulence, as well as the process of formation of resistance to chemotherapeutic drugs. [13]. To date, the range of effective antimicrobial agents against this association of microorganisms remains limited.

It is known that terbinafine-based antifungal drug is widely used due to his high efficiency and low cost.

Terbinafine is an allylamine derivative that has low toxicity, which justifies such using for the treatment of certain forms mycoses. The antifungal effect of terbinafine is due to suppression of squalene epoxidase in the cell membrane of fungi. It leads to a deficiency of ergosterol and intracellular accumulation of squalene, resulting in the death of the pathogens [14].

Benzoyl peroxide is an antiseptic agent, which has been used in the experiment. It has a high keratolytic, anti-inflammatory effect. Its effectiveness has been proven in relation to many microorganisms, as well as to the association of *C. albicans* and *S. aureus*. Preparations containing this substance are used to treat skin diseases. Benzoyl peroxide is one of the promising drugs for the treatment of superficial mycoses. It has nonspecific mechanism of action, like other peroxide compounds. The destruction of the cell wall of microorganism occurs due to oxidation of double bonds in unsaturated fatty acids of membranes. The antifungal activity of benzoyl peroxide is practically not described in the literature. However, there are some reports of successful using of this drug to treat pressure ulcers complicated by fungal infection. In addition, there is information on the development of compositions containing imidazole and benzoyl peroxide [15].

Literature data show that sublethal concentrations of hydrogen peroxide are able to suppress the formation of staphylococcal biofilms by repressing the ica-operon. It leads to a slowdown in the transcription of the ica-ABCD gene, as a result of which the production of the extracellular polysaccharide matrix is suppressed [16]. This could be because benzoyl peroxide has the same inhibitory effect on biofilms containing *S. aureus*.

Thus, the combined using of terbinafine and benzoyl peroxide for the treatment of staphylococcal candidiasis can improve the antimicrobial effect by increasing the oxidation of fatty acids and inhibiting the enzymes of the cell membrane of fungi and bacteria.

The aim of the study was to investigate the antimicrobial activity of a combination terbinafine and benzoyl peroxide on biofilms, including both clinical and reference strains of *C. albicans* and *S. aureus*.

## Materials and Methods

In the process 67 clinical strains of microorganisms were studied: 57 strains of *S. aureus* isolated from patients with various pyoinflammatory diseases in the first 48 hours after hospitalization. Reference strains for the control group (ATCC 25923, ATCC 6538 R). 10 strains of *C. albicans* isolated from the sputum of patients with pneumonia and tracheal lavages and a reference strain (control group) (ATCC 885-653). Isolation and identification of pure cultures of *S. aureus* and *C. albicans* strains was done according to generally accepted microbiological methods, based on morphological, tinctorial, cultural and biochemical properties.

The ability of microorganisms to form biofilms was studied on polystyrene plates for enzyme immunoassay. The formed biofilms were washed and stained with 1% alcohol solution of gentian violet. The optical density of biofilms was measured at a wavelength of 545 nm on a LabLine-90 biochemical analyzer. The measurement of the optical density of biofilms was expressed in optical density units (OD units).

The minimum inhibitory concentration (MIC) of antimicrobial substances on plankton cells and biofilms, which consist of *S. aureus* and *C. albicans* was determined by the method of serial dilutions. MIC is the lowest concentration (in μg/mL) of an antibiotic that inhibits the growth of a given strain of bacteria to provide complete suppression of visible growth of microorganisms. The results were recorded visually, comparing the growth of microorganisms in the presence of antimicrobial substances and with the growth of microorganisms without them. The results were also evaluated by optical density at a wavelength of 545 nm on a LabLine-90 analyzer. The obtained results were compared with the average optical density of the control samples. The viability of microorganisms was determined by counting the number of colony-forming units (CFU) in 1 ml of the culture medium of the experimental samples.

Statistical data processing was carried out according to the generally accepted method. The arithmetic mean and its standard error (M±m) were calculated. Differences were regarded as statistically significant at ($p < 0.05$). The research results were processed using the EXEL and STATISTICA 6 applied programs.

## Research results and their discussion

According to the study, the ability to form biofilms in clinical and reference strains was different (table 1). The average optical density for clinical strains of *S. aureus* was (1.0683±0.006) units. The indicators of the reference strains were lower and the level was (0.0550±0.007) units ($p<0.05$).

The film formation of isolated strains *C. albicans* had indicators of (1.0786±0.006) units. The film formation of reference strains had average rates (0.0650±0.006) units. The highest rates of film formation were determined in the association of microorganism's *C. albicans* and *S. aureus*. So, the average optical density of clinical strains in the association was (1.0892±0.007) units, while in reference strains − (0.0776±0,004) units. The decrease in the optical density of the reference strains is explained by the loss of their virulent properties.

Determination of the minimum inhibitory concentration (MIC) of terbinafine, benzoyl peroxide and their combination on planktonic cells and biofilms of *C. albicans* and *S. aureus* were shown in the (table 2).

Table 1

Determination of the level of biofilm formation by strains of *C. albicans* and *S. aureus* in terms of optical density and CFU

| **Strains** | **Medium optical density test samples (OP unit) λ=545 нм (M±m)** | **CFU×10⁹ per 1 ml of culture medium of the test samples (M±m)** | **Average optical density of control samples (culture medium) (OP unit) λ=545 нм (M±m)** |
|---|---|---|---|
| Clinical strains *S. aureus* | 1.0683±0,006* | 3.5±0.2 | 0.324±0.003** |
| Referent strains *S. aureus* | 0.0550±0,007* | 2.5±0.3 | 0.276±0.006** |
| Clinical strains *C. albicans* | 1.0786±0,006* | 3.6±0.1 | 0.279±0.003** |
| Referent strains *C. albicans* | 0.0650±0,006* | 2.2±0.2 | 0.348±0.004** |
| Clinical strains *C. albicans* + *S. aureus* | 1.0892±0,007* | 4.5±0.1 | 0.0277±0.006** |
| Referent strains *C. albicans* + *S. aureus* | 0.0776±0,004* | 3.5±0.2 | 0.0284±0.007** |

Note. * − significant difference p<0.05; * − significant difference between groups; ** − significant difference with control; the results of studies of 3 repetitions are presented.

Table 2

Determination of the combined effect of terbinafine and benzoyl peroxide on planktonic cells of clinical strains *C. albicans* and *S. aureus* by average optical density (M±m) and CFU

| **MIC of antimicrobial active ingredients for clinical strains (μg/ml)** | **Average optical density strains with antimicrobial substance (OP unit) λ=545 nm** | **CFU per 1 ml of nutrient medium when inoculated from test samples with an antimicrobial substance** | **Control (culture medium *C. albicans* + *S. aureus*) (OP unit) λ=545 nm** | **CFU per 1 ml of culture medium when inoculated from control samples without antimicrobial substances** |
|---|---|---|---|---|
| *S. aureus* benzoyl peroxide (3.1) | 0.0505±0.007 | (3.5±0.2)×105* | 0.0865±0.006 | (3.7±0.1)×10⁶ |
| *C. albicans* terbinafine (4) | 0.0483±0.005 | no growth* | 0.0825±0.004 | (3.3±0.1)×10⁶ |
| *C. albicans* benzoyl peroxide (6.25) | 0.0563±0.006 | (3.4±0.3)×10⁵* | 0.0870±0.003 | (3.8±0.1)×10⁶ |
| *C. albicans* + *S. aureus* terbinafine + benzoyl peroxide (1.3) | 0.0601±0.012 | *S. aureus* (1.5±0.1)×10⁵** *C. albicans* (0.9±0.2)× 10⁵** | 1.0604±0.014 | *S. aureus* (3.5±0.2)×10⁶ *C. albicans* (3.6±0.1)×10⁶ |

Note. * − significant difference p<0.05 with control; ** − the difference is significant with the control and between the action of the combination and the individual active ingredients; the results of studies of 3 repetitions are presented.

The efficacy of benzoyl peroxide on planktonic forms of clinical strains *S. aureus* and *C. albicans* was determined at a concentration of 3.1 μg/ml and 6.25 μg/ml. The concentration of terbinafine for clinical strains of *C. albicans* was 4 μg/ml. When studying the combined action of terbinafine and benzoyl peroxide, a high antimicrobial activity was revealed in relation to the planktonic cells of the association of *C. albicans* and *S. aureus*, the MIC was 1.3 μg/ml.

Microorganisms in the biofilms are highly resistant to antimicrobial agents compared to planktonic cells. To determine the antimicrobial efficacy of this combination against biofilms formed by the association of *C. albicans* and *S. aureus.* The concentration of these substances was increased 10 times (table 3).

The search for an effective therapy for polymicrobial infections is a serious problem. Such traditional therapies are most often directed at individual pathogens of different

Table 3

Determination of the combined effect of terbinafine and benzoyl peroxide on biofilms of clinical strains *C. albicans* and *S. aureus* by average optical density (M±m) and CFU

| **10 MIC of antimicrobial active ingredients for clinical strains (μg/ml)** | **Average optical density of biofilms strains with antimicrobial substance (OD unit) λ=545 nm** | **CFU per 1 ml of nutrient medium inoculated from test samples with an antimicrobial substance** | **Control (culture medium + biofilms *S. aureus*, *S. albicans*) (OD unit) λ=545 нм** | **CFU per 1 ml of nutrient medium inoculated from control samples without antimicrobial substances** |
|---|---|---|---|---|
| *S. aureus* benzoyl peroxide | 1.0088±0.005 | $(4.1±0.3)×10^{8}$* | 1.0865±0.006 | $(3.4±0.2)×10^{9}$ |
| *C. albicans* terbinafine | 0.0715±0.007 | $(2.7±0.2)×10^{8}$* | 1.0935±0.005 | $(3.6±0.1)×10^{9}$ |
| *C. albicans* benzoyl peroxide | 0.0742±0.007 | $(3.2±0.2)×10^{8}$* | 1.0889±0.002 | $(3.5±0.1)×10^{9}$ |
| *C. albicans* + *S. aureus* terbinafine + benzoyl peroxide | 0.0931±0.012 | *S. aureus* $(2.5±0.1)×10^{8}$** *C. albicans* $(1.9±0.2)×10^{8}$** | 1.0984±0.014 | *S. aureus* $(3.5±0.2)×10^{9}$ *C. albicans* $(3.6±0.1)×10^{9}$ |

Note. * – significant difference p<0.05 with control; ** – significant difference with control and between the action of the combination and individual active ingredients; the results of studies of 3 repetitions are presented.

classes [17]. Combination therapy is being explored as a possible alternative to address this problem.

Scientific studies have demonstrated the effectiveness of the synergistic action of anidulafungin in combination with tigecycline against mixed biofilms of *C. albicans* and *S. aureus* in vivo experiments on mice. [18]. It should be noted that essential oils have a pronounced antimicrobial effect. Scientists have shown in vitro the combination of fluconazole and mupirocin with clove oil is 10 times more effective against mixed biofilms than fluconazole or mupirocin alone [19]. The experimental results showed that the combination of fluconazole (8 μg/ml) and minocycline (8 μg/ml) inhibited approximately 80 % of the growth of biofilms formed by resistant strains of *C. albicans* to fluconazole and resistant strains of *S. aureus* to oxacillin [20].

Summing up the results of the study, it was found that the combination of terbinafine and benzoyl peroxide has an increased antimicrobial activity against biofilms of the association of C. *albicans* and *S. aureus*. A decrease in the optical density of biofilms and a decrease in the number of CFU were observed almost 2 times. The obtained data correspond to literature sources. It is confirmed by the concentrations of antimicrobial substances which effective against planktonic forms, have no effect on biofilms. Therefore, their active concentration was increased several times. The effectiveness of such a high concentration has been proven in in vitro experiments, but many questions remain unexplored. Understanding complex interspecies communication and microbial behavior when two or more species exist together can improve our knowledge of polymicrobial infections and provide effective treatment strategies. The findings regarding the combined effect of the antimicrobial substances terbinafine and benzoyl peroxide on the association of *C. albicans* and *S. aureus* make it possible to expand the range of using effective chemotherapeutic agents that affect these pathogens. In the future, these dosage forms can be used for topical application for the prevention or treatment of acute and chronic pyoinflammatory infections on the skin and subcutaneous tissue caused by microorganisms *C. albicans* and *S. aureus*.

## Conclusions

1. The results of the study showed that the clinical strains have a higher ability to form biofilms than the reference strains.

2. The highest optical density values were found in the association of *C. albicans* and *S. aureus*. It means that microorganisms which located in the biofilms are more resistant to antimicrobial substances than planktonic forms.

3. The combination of terbinafine and benzoyl peroxide had a high antimicrobial activity to the planktonic cells of association of *C. albicans* and *S. aureus*, than to biofilms (p<0.05).

4. A decrease in the optical density of biofilms and a decrease in the number of CFU were observed almost 2 times. So the combination of terbinafine and benzoyl peroxide had antimicrobial activity against the biofilms of the association of *C. albicans* and *S. aureus*.

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# СИНЕРГІЧНА АНТИМІКРОБНА АКТИВНІСТЬ ПОЄДНАННЯ ТЕРБІНАФІНУ ТА БЕНЗОЇЛПЕРОКСИДУ ПРОТИ CANDIDA ALBICANS ТА STAPHYLOCOCCUS AUREUS

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*РЕЗЮМЕ. Мета роботи – дослідити здатність мікроорганізмів C. albicans і S. aureus утворювати біоплівки, а також оцінити їх чутливість до комбінації тербінафіну та пероксиду бензоїлу.*

***Матеріали і методи.*** *Здатність мікроорганізмів утворювати біоплівки та антимікробну дію досліджуваних препаратів оцінювали на полістирольних пластинах для імуноферментного аналізу. Оптичну густину (ОГ) біоплівок вимірювали за довжиною хвилі 545 нм на біохімічному аналізаторі. Життєздатність мікроорганізмів визначали шляхом підрахунку кількості колонієутворювальних одиниць (КУО) в 1 мл культурального середовища з дослідними штамами.*

***Результати.*** *Середня оптична щільність біоплівок становила (1,0892±0,006) од., а ОГ для клінічних ізолятів – (0,0776±0,004) од. Доведено, що комбіна-*

*ція антимікотичної речовини тербінафіну та антисептика бензоїлпероксиду мала високу активність до асоціації C. albicans та S. aureus у концентрації 1,3 мкг/мл.*

***Висновок.** Дослідження показало, що здатність до утворення біоплівок у клінічних штамів мікроорганізмів значніша, ніж в еталонних штамів. Найбільша швидкість утворення біоплівки виявлена в асоціації мікроорганізмів C. albicans і S. aureus. Комбінація ліків також ефективно діяла на планктонні форми бактерій, а також на мікроорганізми, мобілізовані в біоплівках. Про це свідчить зниження ОГ та 2-разове зменшення КУО.*

***Ключові слова:** полімікробна інфекція, біоплівки, комбінована дія.*

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