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Effects of Miramistin and Phosprenil on Microbial Biofilms

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Effects of Miramistin and Phosprenil on biofilms of *S. pyogenes*, *S. aureus*, *E. coli*, *L. acidophilus*, and *L. plantarum* were studied. Significant differences in the effects of these substances on mature biofilms of microorganisms and the process of their formation were observed. Miramistin had significant inhibiting effects on the forming of biofilms and on the formed biofilms of all studied microorganisms. Treatment with Miramistin inhibited biofilm formation by 2-3 times compared to the control. This effect was found already after using of Miramistin in the low doses (3.12 μg/ml). Inhibition of the growth of a formed biofilm was observed only after treatment with Miramistin in the high doses (25-50 μg/ml). Phosprenil in the high doses (15-30 mg/ml) inhibited the forming of biofilms, especially the biofilms of *S. pyogenes* and *L. plantarum* (by 3-4.5 times). Treatment of formed biofilms with the agent in doses of 6.0 and 0.6 mg/ml was associated with pronounced stimulation of its growth in *S. pyogenes*, *S. aureus*, and *L. acidophilus*.

Key Words: *microorganisms; biofilms; Miramistin; Phosprenil*

Biofilms are known to be a defense method, which allows bacteria to growth and reproduce under unfavorable conditions. The process of biofilm formation includes the following stages: adhesion of microorganisms to the surface, colony generation, maturation of a biofilm with matrix formation, and detachment of the attached cells with the following settlement in other places. Adhesion to various substrates (biotic — tissues of humans and animals, and abiotic — glass, plastic, or metal) is an important property of biofilms. The structure of a biofilm includes bacterial clusters attached to the surface and each other and covered with a biopolymer matrix on the outer side. The matrix consists of proteins, nucleic acids, lipids, but exogenous polysaccharides are the main component. The matrix protects a biofilm from damaging factors including the factors of innate and acquired immunity of the macroorganism, medicinal substances, and others. In addition, the microorganisms in the film differ from plankton cells by phenotype and expression of specific genes. This form of bacteria produces huge problems for therapists, as the ability to form biofilms is one of the main mechanisms of chronic infections. The biofilm is pierced by a net of channels providing nutrition for the cells and signaling, which is necessary for the functioning of Quorum sensing, *i.e.* intercellular interactions [2,6,9].

Production of new antibiotics and enlargement of their treatment areas has been followed by the appearance of new strains of pathogenic bacteria resistant to various antibiotics. Fast spreading of genes of multiply resistance has been promoted by the migrating gene elements, *e.g.* plasmids and phages, which include the

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genes in their composition and transfer them to other bacteria [8]. Biofilms serve as another factor determining increased resistance of bacteria to antibiotics. Biofilms are significantly less sensitive to antibiotics than bacteria in plankton form. It is shown that despite the high sensitivity of *S. pyogenes* to penicillin, some patients are resistant to the treatment with this and other antibiotics [1,5]. Thus, a search of alternative methods for treatment of bacterial infections and testing of various agents damaging biofilms or inhibiting their growth, particularly of plant origin, are of specific interest. Positive results were obtained after treatment with usnic acids of the lichens, manuka honey, and limonene from the citrus plants, which have antibacterial properties [7,10,13].

We studied the effects of two medicinal substances Miramistin and Phosprenil on the biofilms of various microorganisms. Miramistin has a wide spectrum of antibacterial properties including gram-positive microorganisms. Phosprenil (PP) isolated from the needles of coniferous plants is a natural organic substance of the group of polyprenyl phosphates, which have high biological and anti-inflammatory activity. Antiviral activity of PP has also been shown [12].

Here we studied the effects of Miramistin and PP on the process of forming of biofilms and already formed mature biofilms of various microorganisms.

MATERIALS AND METHODS

The strains with high abilities to produce biofilms were used: *S. pyogenes* — a strain 30M from the Prague collection and a clinical strain 36B, *S. aureus* — a strain from the collection ATCC 6538R and a clinical strain 010Ng, *E. coli* — a strain from the collection ATCC 25922 and a clinical strain Ds 205, *L. acidophilus* — strain NK1, and *L. plantarum* — the strain 30. Culturing was performed in Todd-Hewitt broth (streptococcus), Nutrient broth (*staphylococcus* and *E. coli*), and MRS medium (*lactobaccili*) for 18 h at 37°C.

Biofilm formation was studied as described elsewhere [11] in sterile polystirol 96-wells Costar plates. Daily culture of a studied strain was diluted in the appropriate medium to a concentration corresponding to 10.0 of McFarland turbidity standard (Den-1 McFarland Densitometre, Biosan). Then, the suspensions of streptococcus and lactobacillus were diluted with the medium in the ratio 1:40, and suspensions of staphylococcus and *E. coli* were diluted in the ratio of 1:100. Miramistin and PP in the various concentrations were added to vials with the microbial suspension, mixed, and replaced to the wells of a plate per 100 µl. The plates were placed into a thermostat for 2 days at 37°C and 5% CO_2. A suspension of bacterial cells without the test substances served as a positive control. Nutrient medium was used as a negative control. The substances in various concentrations were added to the wells in a volume of 100 µl after removal of plankton cells and washing the wells and incubated for 24 h at 37°C in order to investigate the effects of the agents on the formed biofilm. Crystal violet (100 µl of 1% water solution) was added to the washed wells for biofilm staining. The dye was removed after 5 min, the wells were washed with distilled water, and filled with 100 µl of 96° ethanol. The intensity of staining of the ethanol solution was estimated using iEMS Reader MF spectrophotometer (LabSystems) at λ=540 nm. PP in concentrations of 30-0.06 mg/ml and Miramistin in doses of 50-1.56 µg/ml were used.

The results were analyzed using Student's *t* test. The results are presented as the mean of 4 experiments ($M \pm m$).

RESULTS

Significant deceleration of biofilm growth in comparison to the control was observed after the treatment with Miramistin in low concentrations (3.12 µg/ml). Under these conditions, the intensity of biofilm generation decreased by 1.5-2 times in the museum strain of streptococcus 30M and by 2-3 times in the clinical strain 36B ($p<0.01$; Fig. 1). Miramistin (12-25 µg/ml) induced a decrease in the intensity of biofilm production by 2.5-1.6 times even in the strains of *E. coli*, which is characterized by a low ability to produce biofilms. The most pronounced reduction in biofilm growth was found in *S. aureus* ATCC strain: by 3.4 times after treatment with Miramistin in a dose of 3.12 µg/ml and by 6.5 times after treatment with the higher doses of the agent ($p<0.01$).

Far higher concentrations of Miramistin (12.5-50.0 µg/ml) were needed to inhibit the growth of

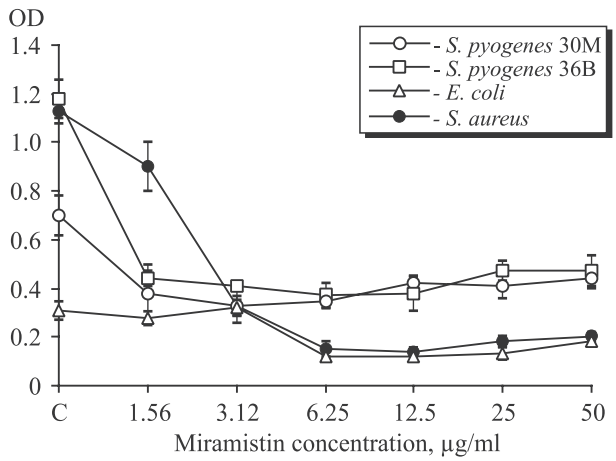

Fig. 1. Effects of Miramistin on the forming of microbial biofilm. C: positive control.

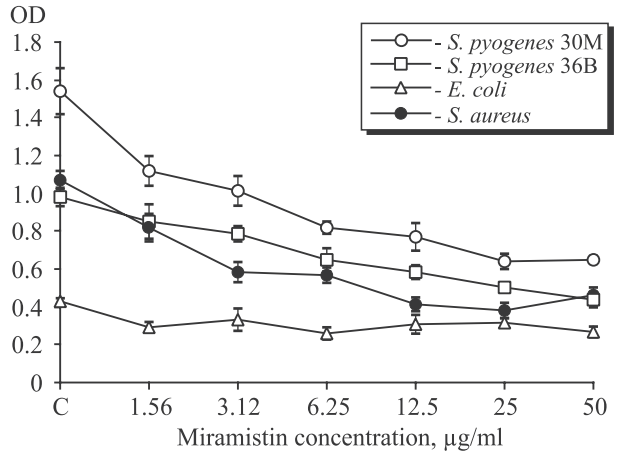

Fig. 2. Effects of Miramistin on the formed biofilm. C: positive control.

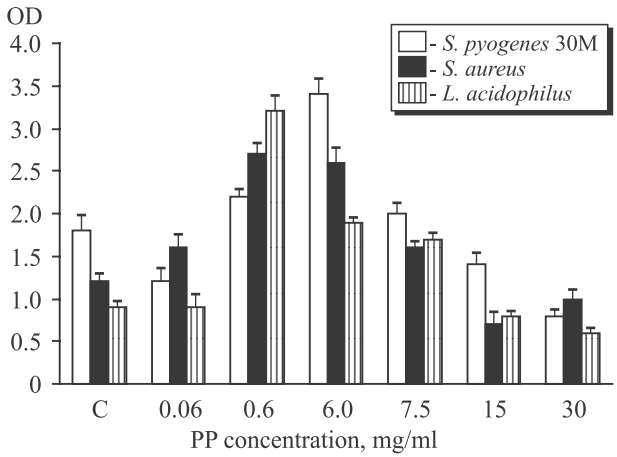

Fig. 3. Effects of PP on the formed biofilm. C: positive control.

formed biofilm (Fig. 2). The intensity of film production under these conditions decreased by 2.0-2.3 times in *S. pyogenes*, by 1.6 times in *E. coli*, and by 2.6 times in *Staphylococcus*.

In the next experimental series, the effects of PP on the forming of biofilms and already formed biofilms were studied. The intensity of biofilm production in *Streptococcus* significantly reduced after the treatment with PP in high doses (30 and 15 mg/ml): by 3.0-3.8 times in 30M strain and by 2.7 times in 36B strain compared to the positive control (Table 1). The agent in doses of 6.0 and 0.6 mg/ml induced a slight increase in the optical density indicating a slight rise in the biofilm growth. The growth of *Staphylococcus* was inhibited by about 2 times after treatment with PP in concentrations of 30-7.5 mg/ml. The growth of *E. coli* biofilm was inhibited by 1.8-2 times after application of PP in a dose of 30 mg/ml. High doses of PP did not affect the formation of biofilm by *L. acidophilus*, but they affected the growth of biofilms of *L. plantarum* (decreased by 4.5 times).

Therefore, high doses of PP normally inhibited the forming of biofilms. Maximal effect was observed after the treatment of *Streptococcus* strain 30M (by 3.8 times) and *L. plantarum* strain (4.5 times). The treatment with PP ~2-fold inhibited biofilm formation by both strains of *Staphylococcus* and *E. coli*. The degree of inhibition of the growth by PP dose-dependently decreased. It should be noted that PP in doses of 0.6-0.06 mg/ml stimulated the growth of ATCC strain of *E. coli* and *L. acidophilus*. The treatment with the agent in a dose of 0.6 mg/ml induced an increase in the intensity of biofilm production in *E. coli* by 2 times and in *L. acidophilus* by 9.25 times, compared to the positive control. Thus, PP in doses of 6-0.06 mg/ml stimulated biofilms formation by some strains.

TABLE 1. Effects of PP on Biofilm Forming by Various Microorganisms (OD; *M±m*)

PP concentration, mg/ml	*S. pyogenes*		*S. aureus*		*E. coli*		*Lactobacillus*	
	30M	36B	ATCC	010Ng	ATCC	Ds 205	Acidophilus	Plantarum
Positive control	0.76±0.08	1.05±0.04	1.12±0.12	1.14±0.09	0.31±0.09	0.37±0.02	0.27±0.03	1.04±0.08
Negative control	0.35±0.02	0.35±0.05	0.12±0.02	0.11±0.01	0.23±0.02	0.14±0.03	0.24±0.04	0.17±0.01
30	0.22±0.09	0.38±0.01	0.51±0.09	0.51±0.08	0.17±0.00	0.19±0.01	0.28±0.04	0.23±0.02
15	0.21±0.05	—	0.68±0.02	0.41±0.03	0.24±0.02	0.37±0.06	0.30±0.01	0.26±0.03
7.5	0.24±0.04	—	0.61±0.03	0.67±0.06	0.49±0.09	0.47±0.11	0.33±0.02	0.35±0.12
6.0	0.38±0.02	0.42±0.07	0.72±0.06	0.67±0.04	0.41±0.08	0.27±0.03	0.72±0.07	0.52±0.04
0.6	0.51±0.02	0.63±0.09	0.44±0.00	0.21±0.01	0.65±0.07	0.34±0.03	2.50±0.14	0.71±0.06
0.06	0.41±0.03		0.75±0.05	0.97±0.07	0.29±0.01	0.35±0.04	0.87±0.08	1.02±0.05

Note. "—", the concentration was not studied. The levels are significant compared to the positive control at $p<0.01$.

A strong stimulating effect of PP on the formed biofilm of some strain was observed. The most pronounced effects were found after the treatment of *Streptococcus* strains 30M and 36B, *Staphylococcus* strain 010Ng, and *L. acidophilus* strain NK1 with PP in doses of 6.0 and 0.6 mg/kg (Fig. 3).

PP in a concentration of 6 mg/ml stimulated the growth of biofilm of *Streptococcus* strain 30M by 3.4 times compared to the control. An increase in biofilm growth by 2.6-2.7 times was found in a *Staphylococcus* strain after the application of PP in doses of 6.0 and 0.6 mg/ml. Strong stimulation of biofilm growth was noted in *L. acidophilus* strain NK1: by 3.2 and 1.9 times after the treatment with PP in concentrations of 0.6 and 6 mg/ml, respectively ($p<0.01$).

Our experiments revealed significant differences in the effects of Miramistin and PP on the biofilms produced by various microorganisms. Miramsitin had significant inhibiting effects on the forming of biofilms and already formed ones. However, higher doses of the substance (50-25 μg/ml) were needed for the inhibition of formed biofilm. Effects of PP in the biofilms were contradictory. PP in high doses (30-15 mg/ml) inhibited biofilm formation, especially in *S. pyogenes* and *L. plantarum*. However, the agent in doses of 6.0 and 0.6 mg/ml significantly stimulated biofilm formation by *S. pyogenes*, *S. aureus*, and *L. acidophilus*. No stimulating effects on biofilms formed by *E. coli* and *L. plantarum* were noted..

PP is a member of isoprenoid family, which is a part of all living cells including microorganisms. They contribute to the biosynthesis of bacterial glycoproteins and peptidoglycans, maintain energy balance in cells, promote forming of cell membranes and signaling. It cannot be excluded that microbial cells might use PP at various stages of metabolic processes [12]. The stimulation of biofilm production by some *Lactobacilli* is of specific interest. It is known that *Lactobacilli* contribute to the forming of intestinal microflora. They have antagonistic activity towards pathogenic microorganisms by concurrent binding to the surface receptors of epithelial cells and releasing the probiotic properties. The surface lectins of *Lactobacilli* can affect the process of generation of biofilms of gram-positive bacteria at various stages of development, which might result in disorganizing of the films [3,4]. Thus, on one hand, the search for substances disintegrating the biofilms of pathogenic microorganisms is of specific interest, and on another hand, a search of substances stimulating generation of biofilms of useful bacteria also should be performed.

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