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THE INHIBITORY AND DESTRUCTIVE ACTION OF THE SILVER NANOPARTICLE PREPARATION ON BIOFILMS FORMED BY CLINICALLY RELEVANT MICROORGANISMS

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AIM: to obtain and investigate the activity of silver nanoparticles stabilized with arabinogalactan in relation to clinically relevant strains of film-forming microorganisms.
MATERIALS AND METHODS: silver nanoparticles were obtained by reduction from silver nitrate in the presence of arabinogalactan with additional stabilization with dioctyl sodium sulfosuccinate. The shape and size of the nanoparticles were determined by the method of transmission electron microscopy, the zeta potential by the method of electrophoretic light scattering. The study of the effect of the nanoparticles on biofilm formation was carried out on 17 clinically relevant strains of bacteria isolated from blood culture and the clinical biomaterial of postoperative patients.
RESULTS: the silver nanoparticles with an average diameter of 11.4 nm and a zeta potential of –24 mV were obtained. The minimum inhibitory concentration of the nanoparticles in relation to planktonic form of bacteria was 120 mg/ml; the use of the drug at a concentration of 100 mg/ml reduced the amount of CFU by 7 orders of magnitude compared with the initial culture. The study of the effect of silver nanoparticles on the formation of biofilms showed that, in the presence of the drug, the growth of biofilms was significantly reduced; at a drug concentration of 150 mg/ml, the growth of bacterial films was completely suppressed. Incubation of the formed daily biofilms with the silver nanoparticles in the concentration range from 150 to 120 mg/ml for 48 h resulted in the partial or complete destruction of the biopolymer matrix.
CONCLUSION: the studied preparation of silver nanoparticles has a great potential for use in the treatment of infectious diseases caused by biofilm forming microorganisms.

[Keywords: biofilms, silver nanoparticles, antimicrobial activity, clinical isolates,catheter-associated infection, postoperative wounds]

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INTRODUCTION

One of the serious problems of modern medicine is the multiple drug resistance of pathogens significantly reducing the effectiveness of antibiotic therapy [10,24]. Polyresistance is a complex mechanism, the specific implementation of which depends on the characteristics of the organism, the strain of infectious disease, the antibiotic used and other factors [2]. The search for new drugs, including nanotechnologies that reduce the risk of development or overcome multiple drug resistance, and, consequently, significantly increase the effectiveness of treatment of dangerous infectious diseases, is an extremely important task [6].

Many purulent inflammatory diseases and postoperative complications are associated with antibiotic-resistant film-forming microorganisms, which are much more resistant than plankton forms [4]. The formation of biofilms by pathogenic microorganisms contributes to infectious lesions of both human organs and artificial implants. Biofilms are structures formed by microbial communities on the interface of phases, for example, liquid and solid, liquid and gaseous, etc. [13,20]. It is important to note that biofilms can be formed by bacteria of one species as well as represent a community, including several species of bacteria and other microorganisms. The microbial community itself consists of cells enclosed in an extracellular polymer matrix (exopolymer matrix) synthesized by them.

Biofilms play an important role in the process of transition of infectious disease from acute to chronic phase, being the cause of significant difficulties in the effective treatment of a large number of pathologies. In this regard, the search for means preventing the formation of biofilms and affecting bacteria inside them is an important task of modern antimicrobial therapy [23].

As a promising means of treatment of infectious and purulent-inflammatory diseases as an alternative to antibiotics, the properties of silver metal nanoparticles that exhibit a wide range of antimicrobial activity in the absence of resistance to them are being actively studied [7,21].

The toxic effect on bacterial cells is significantly determined by the size, shape, concentration, nature of the stabilizer of nanoparticles, as well as their ability to generate reactive oxygen species [9,15]. Silver nanoparticles are able to sorb on the surface of the bacterial cell, have a damaging effect on the plasma membrane and cause cell death.

Silver nanoparticles can also penetrate into bacterial cells and have a damaging effect on intracellular structures, ribosomal subparticles, nucleic acids and proteins [7,16,26].

Among the mechanisms of the toxic effect of silver nanoparticles on bacteria, their most important property is the ability to destroy the exopolymer matrix of biofilm [11,25]. This property acquires additional significance on the basis of the fact of the absence of nanosilver resistance in biofilms produced by multiresistant bacteria [19,22].

The nature and effectiveness of the toxic action of silver nanoparticles against film-forming microorganisms is largely determined by the nature of stabilizing biopolymers. The cytotoxic activity of silver nanoparticles stabilized with citrate [12], cyclodextrin [14], polyvinylpyrrolidone [5] and starch [17] against *Pseudomonas aeruginosa, Staphylococcus aureus, Shigella flexneri, Streptococcus pneumoniae, Bacillus subtilis, Escherichia coli*, etc. has been demonstrated.

One of the promising biopolymers that can be used for the recovery of metal nanoparticles from silver salts and their stabilization is natural water-soluble, non-toxic, easily accessible arabinogalactan from Siberian larch (*Larixsibirica L.)* and garden purslane (*Portulaca oleracea*) [3,18]. Steric stabilization of the surface of nanoparticles with arabinogalactan can significantly increase their stability and, while maintaining high antimicrobial activity, reduce toxic effects on the human body.

Thus, the problem of combating resistant film-forming bacteria dictates the need to develop effective drugs that can destroy the exopolymer matrix of biofilms and effectively affect bacterial cells. Development of approaches to the production and study of antimicrobial activity of silver nanoparticles against film-forming clinical strains of microorganisms is an urgent task.

In this work, we have obtained and characterized arabinogalactan stabilized silver nanoparticles. Their activity against clinically significant strains of film-forming microorganisms was studied.

MATERIALS AND METHODS

Preparation and characteristics of silver nanoparticles

For the synthesis of silver nanoparticles silver nitrate, ammonium hydroxide (27%), sodium dioctyl sulfosuccinate (Aerosol-OT, or bis (2-ethylhexyl) sulfosuccinate, sodium salt) (Labtex, Russia), arabinogalactan (Fluka) were used.

Synthesis of silver metal nanoparticles was performed by reduction of silver nitrate in alkaline medium in the presence of arabinogalactan. A solution of silver nitrate was added to 0.2% arabinogalactan solution heated to 90°C with intensive stirring. The silver reduction reaction was carried out for 40 minutes at the same temperature and pH≥10.0, followed by the addition of sodium dioctyl sulfosuccinate and gradual cooling of the solution to room temperature.

Electro kinetic potential of silver nanoparticles was determined by electrophoretic light scattering on the analyzer Photocor compact Z (Russia).

Studies using transmission electron microscopy were performed using a microscope LEO 912 AB (Carl Zeiss, Germany). Studies were carried out at an accelerating voltage of 100 kV. To prepare the samples, a drop of sol was applied to copper meshes with a diameter of 3.05 mm, covered with a thin polymer film-substrate, and dried at room temperature.

The distribution of silver nanoparticles size was determined by processing the obtained micro-

Table 1. *Strains of microorganisms used in the work*

The name of the microorganism	The number of the strain	Location of extraction
Acinetobacter baumanii	152	mucus
Acinetobacter baumanii	480	blood
Acinetobacter lwoffii	679	drainage from the abdomen
Acinetobacter lwoffii	756	drainage from the wound
Acinetobacter lwoffii	775	drainage from the wound
Escherichia coli	5	faeces
Escherichia coli	32	drainage from the abdomen
Escherichia coli	40	drainage from the abdomen
Escherichia coli	84	drainage from the abdomen
Escherichia coli	317	blood
Klebsiella pneumoniae	5	faeces
Klebsiella pneumoniae	29	drainage from the pleural cavity
Klebsiella pneumoniae	50	drainage of fistula
Klebsiella pneumoniae	107	drainage from the abdomen
Klebsiella pneumoniae	458	blood
Pseudomonas aeruginosa	1000	blood
Pseudomonas aeruginosa	15	drainage from the abdomen

photographs using the program analysis of the optical images of UTHSCSA Image Tool 3.00.

Strains of microorganisms

Clinically significant strains of microorganisms that were most commonly found as pathogens of nosocomial and chronic infections were selected for the study; these bacteria were also characterized by resistance to beta-lactam antibiotics (Table 1).

The studied bacteria were isolated from hemoculture and various clinical biomaterials of the patients undergoing surgical treatment for coloproctological diseases. 17 strains of different species were studied to identify possible differences in drug interactions.

Study of the effect of silver nanoparticles on clinical isolates

1) The effect of the preparation of silver nanoparticles on the plankton form of microbial cells. To determine the sensitivity to silver nanoparticles, the minimum inhibitory concentrations (MIC) were measured in 96-well polystyrene plates using the method of successive two-fold dilutions.

The bacterial suspension was prepared in saline solution; the density of the suspension corresponded to 1 McF (3.3×108 CFU/ml). Dilutions of the preparation of silver nanoparticles were carried out in the cardio cerebral extract in the concentration range from 150 to 1.6 μg/ml. Bacterial suspensions with the drug were incubated in different concentrations for 48 hours at 37°C. Quantitative assessment of inhibition of microbial growth was carried out using the method of serial dilutions followed by sowing on a dense nutrient medium (trypton-soy agar) and counting CFU/ml after incubation at 37°C for 24 hours.

2) Inhibition of bacterial biofilm formation by preparation of silver nanoparticles.

According to the method described above, bacterial suspensions from daily cultures with a density of 1.0 McF were prepared with the addition of silver nanoparticles in different concentrations. 1 ml of the suspension was applied to the cover glasses, kept for 4 hours to fix the cells, and then were added 5 ml of medium for biofilmogenesis (pepton-yeast extract) and the glasses were incubated in a thermostat for 48 hours at 37°C.

3) Destruction of daily biofilms by preparation of silver nanoparticles.

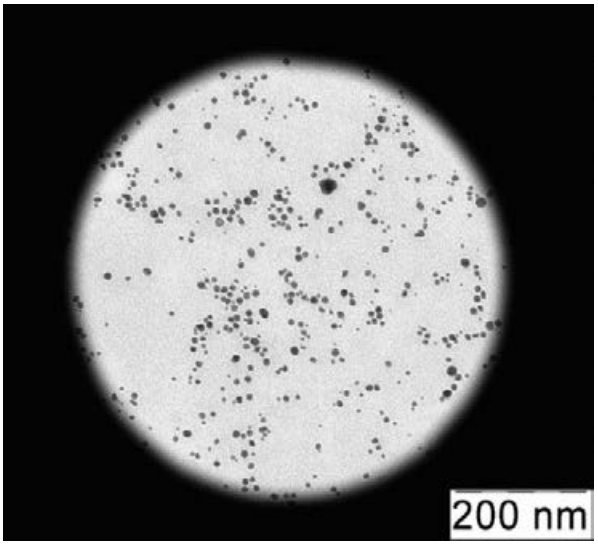

Figure 1. *Image of silver nanoparticles obtained by transmission electron microscopy*

Table 2. *The values of CFU/ml at different concentrations of the preparation of silver nanoparticles (the final concentrations of the preparation of nanoparticles in the resulting solution are given)*

Strain	The indicator CFU/ml			
	Control	Preparation of nanoparticles		
		120 μg/ml	100 μg/ml	75 μg/ml
A.baumanii 152	$5,60\times10^9$	0	30	$8,64\times10^7$
A.baumanii 480	$6,03\times10^9$	0	37	$4,78\times10^7$
A.lwoffii 679	$4,21\times10^9$	0	19	$7,84\times10^7$
A.lwoffii 756	$5,39\times10^9$	0	10	$6,02\times10^7$
A.lwoffii 775	$4,45\times10^9$	0	19	$6,75\times10^7$
E.coli 5	$2,52\times10^9$	0	12	$9,24\times10^7$
E.coli 32	$3,42\times10^9$	0	10	$8,73\times10^7$
E. coli 40	$4,11\times10^9$	0	31	$1,34\times10^8$
E.coli 84	$1,34\times10^9$	0	27	$7,83\times10^7$
E.coli 317	$2,63\times10^9$	0	9	$2,42\times10^8$
K.pneumoniae 5	$3,70\times10^9$	0	56	$3,85\times10^8$
K. pneumoniae 29	$2,69\times10^9$	0	24	$7,96\times10^8$
K.pneumoniae 50	$3,35\times10^9$	0	36	$8,49\times10^8$
K.pneumoniae 107	$3,57\times10^9$	0	61	$4,73\times10^8$
K.pneumoniae 458	$4,16\times10^9$	0	48	$6,45\times10^8$
P.aeruginosa 1000	$2,80\times10^9$	0	29	$7,04\times10^7$
P.aeruginosa 15	$1,13\times10^9$	0	35	$1,28\times10^8$

1 ml of bacterial suspension of 1.0 McF was applied to the cover glasses and kept for 4 hours for fixing cells, after which were added 5 ml of medium for biofilmogenesis and the glasses were incubated for 24 hours at 37°C. On the glasses with the developed biofilm were applied to 1 ml of the test drug in various concentrations and they were incubated for a further 48 hours.

Biofilms were stained with alcian blue and microscopy was performed in a light microscope with a resolution of ×1000. The degree of biofilm formation was assessed by the developed conditional 4-point scale based on the nature of growth and the degree of coating of glass with bacterial film [1]. As a control, biofilm cultures were used, which were obtained under identical conditions, but without the addition of nanoparticles.

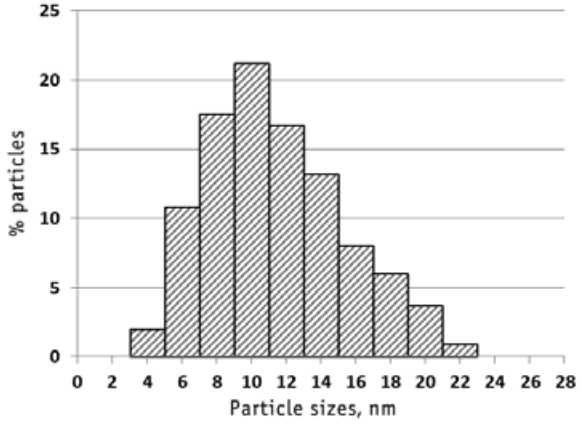

Figure 2. *Histogram of distribution of stabilized silver nanoparticles by size*

RESULTS AND DISCUSSION

Silver sol was obtained by reducing silver nitrate using arabinogalactan, which simultaneously acted as a reducing agent and stabilizer of nanoparticles. To increase the stability of silver sol to the obtained preparation was added dioctylsulfosuccinate sodium. According to transmission electron microscopy, the preparation contained nanoparticles of spherical silver (Fig. 1). The average diameter of nanoparticles was 11.4 nm (Fig. 2); Zeta potential – 24 mV.

At the first stage, the effectiveness of the preparation of nanoparticles on plankton cultures of microorganisms was studied. In the presence of the drug at a concentration of 75 μg/ml, the CFU/ml index in the case of all studied strains was 1-2 orders of magnitude lower compared with the control; at lower concentrations of the drug, the plankton growth remained unchanged. With an increase in the concentration of nanoparticles to 100 μg/ml, a sharp decrease in CFU/ml by 7 orders of magnitude was recorded (Table 2), which indicates the high bactericidal ability of silver nanoparticles. At a concentration of 120 μg/ml of plankton growth of microorganisms was not observed.

The obtained results indicate that the minimum inhibitory concentration (MIC) of the preparation of nanoparticles is 120 μg/ml. It should be noted

Table 3. *Inhibition of biofilm formation of clinical isolates of microorganisms by preparation of silver nanoparticles*

Strain	Degree of biofilm formation			
	Control	Silver nanoparticles		
		150 μg/ml	120 μg/ml	75 μg/ml
A. baumanii 152	4	~0	<1	2
A. baumanii 480	3	~0	<1	2
A. lwoffii 679	3	~0	<1	2
A. lwoffii 756	3	~0	<1	2
A. lwoffii 775	3	~0	<1	2
E. coli 5	3	~0	<1	2
E. coli 32	3	~0	<1	2
E. coli 40	3	~0	<1	2
E. coli 84	3	~0	<1	2
E. coli 317	3	~0	<1	2
K. pneumoniae 5	4	~0	<1	3
K. pneumoniae 29	3	~0	<1	2
K. pneumoniae 50	3	~0	<1	2
K. pneumoniae 107	4	~0	<1	3
K. pneumoniae 458	4	~0	<1	3
P. aeruginosa 1000	2	~0	<1	2
P. aeruginosa 15	3	~0	<1	2

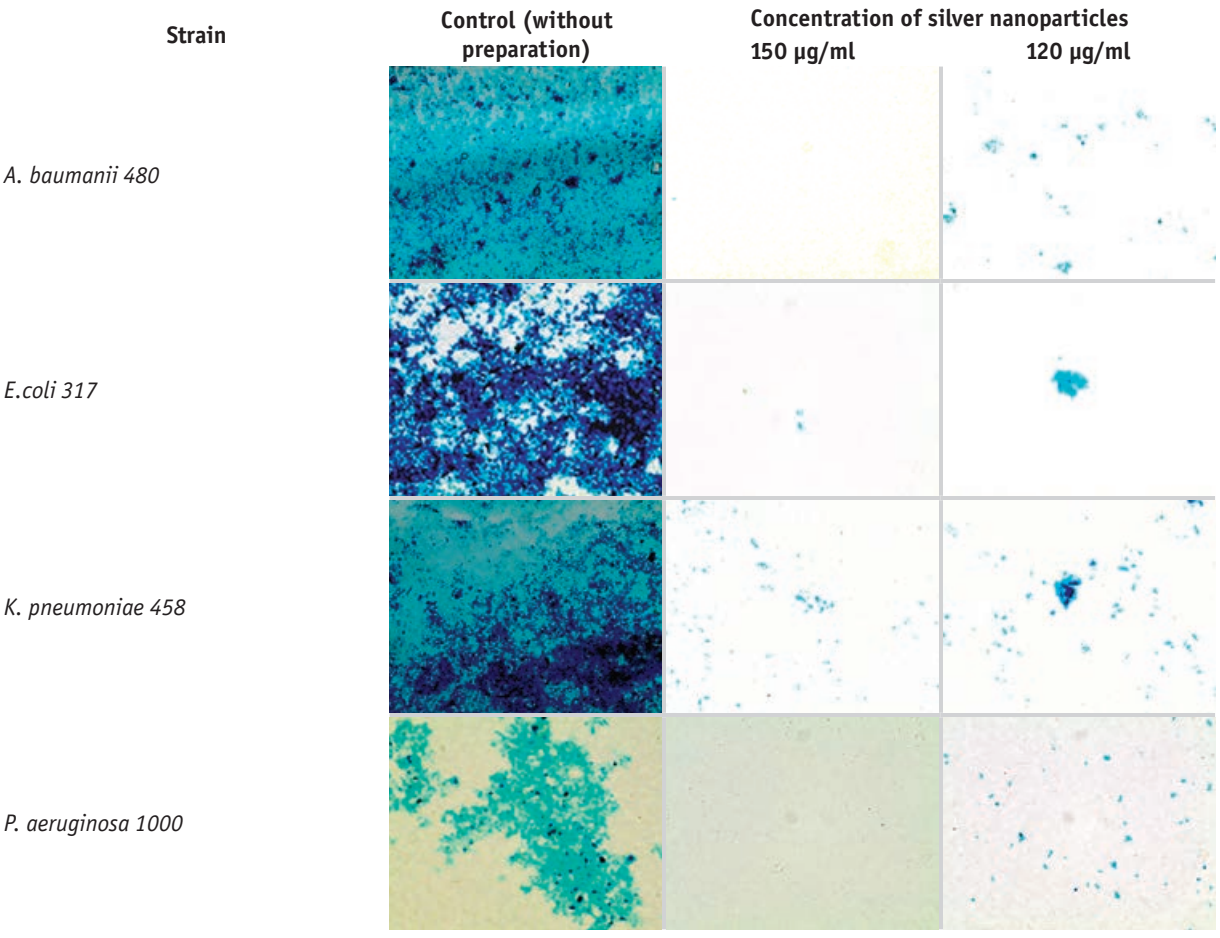

Figure 3. *Inhibition of biofilm formation of some strains of microorganisms after 48 hours of incubation with nanoparticle preparations*

Table 4. *Destruction of daily biofilms of clinical isolates of microorganisms by preparation of silver nanoparticles*

Strain	Degree of biofilm formation			
	Control	Silver nanoparticles		
		150 μg/ml	120 μg/ml	75 μg/ml
A. baumanii 152	4	~0	<1	3
A. baumanii 480	4	~0	<1	2
A. lwoffii 679	4	~0	<1	3
A. lwoffii 756	4	~0	<1	3
A. lwoffii 775	4	~0	<1	3
E. coli 5	4	~0	<1	2
E. coli 32	4	~0	<1	2
E. coli 40	4	~0	<1	4
E. coli 84	4	~0	<1	2
E. coli 317	4	~0	<1	2
K. pneumoniae 5	4	~0	<1	3
K. pneumoniae 29	3	~0	<1	2
K. pneumoniae 50	3	~0	<1	2
K. pneumoniae 107	4	~0	<1	3
K. pneumoniae 458	4	~0	<1	3
P. aeruginosa 1000	3	~0	<1	2
P. aeruginosa 15	4	~0	<1	3

that different representatives of the same kind of microorganisms in interaction with the preparation demonstrate similar reactions (Table 2).

Next, we investigated the effect of the preparation of silver nanoparticles on the biofilm formation. The studied strains of bacteria in the control samples, without the influence of inhibitory factors, for 48 hours formed biofilms of 3-4 degrees according to our evaluation scale (Fig. 3). In the presence of silver nanoparticles, the process of the biofilm growth was significantly decreased at concentrations ranging from 120 μg/ml and above and was evaluated as following the first degree (Table 3). When the preparation of nanoparticles was added at a concentration of 150 μg/ml, the growth of bacterial films was completely suppressed (Fig. 3).

Antibacterial agents that are effective in inhibiting the unformed biofilm can often be ineffective against microorganisms already enclosed in a film matrix. In our study, incubation of formed daily biofilms with the preparation of silver nanoparticles in the concentration range from 150 to 120 μg/ml for 48 hoursled to partial or complete destruction of the biopolymer matrix (Table 4).

CONCLUSION

The obtained results show that the preparation of silver nanoparticles stabilized by the biopolymer arabinogalactan exhibits antibacterial activity against plankton cultures of all studied strains, suppresses the process of film formation, and also causes the destruction of formed biofilms in the same concentration range. Thus, the studied drug has great potential for use in the treatment of infectious diseases caused by film-forming microorganisms, as it will have a complex effect on them.

The authors declare no conflicts of interest.

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