ЕКСПЕРИМЕНТАЛЬНА ФАРМАКОЛОГІЯ

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THE ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES *IN VITRO*

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Key words: silver nanoparticles; antimicrobial activity; spectrum of bacteria

Silver nanoparticles possess a high potential as an antimicrobial substance against a wide spectrum of bacteria, including antibiotic-resistant strains. Antimicrobial properties of silver nanoparticles with 30 nm in diameter synthesized according to the original protocol have been determined in this study. In in vitro study using the serial dilutions method in the solid medium the minimal inhibition concentration (MIC) of silver nanoparticles against such test-strains as Staphylococcus aureus MRSA ATCC 43300, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 2592, Shigella sonnei, Salmonella typhimurium 144 was equal to 33.46 μg/ml. MIC against B. subtilis ATCC 6633 was 133.8 μg/ml. The antimicrobial activity of silver nanoparticles has been studied on clinical isolates with multiple drug resistance isolated from wounds, urine, endocervical and faucial scrapings in surgical patients with Klebsiella ozaenae 4348, Citrobacter freundii 4369, Escherichia coli 4358, Enterobacter aerogenes 2476, Proteus mirabilis 4363, Staphylococcus aureus 4312 and Pseudomonas aeruginosa 283. The total inhibition of the microorganisms growth under the action of both doses of silver nanoparticles studied – 10 μg and 20 μg has been observed.

The search for effective antimicrobial substances is an important task of pharmacology nowadays. The appearance of antibiotic-resistant bacterial strains requires the use of antimicrobial agents with principally new properties compared to traditional drugs, which are able to overcome more successfully the resistance of the causative agents of certain diseases. Metal nanoparticles, and especially silver nanoparticles are in the focus of attention of researchers. It is known that silver nanoparticles are characterized by a pronounced antimicrobial activity. Based on them medicines in the form of creams [6, 9], gels [14], as well as such medical products as catheters [20] and dressings [8, 11] have been developed and introduced into practice. At the Pharmacology Department of O.O.Bohomolets National Medical University the research of pharmacological and toxicological properties of different metal nanoparticles (copper, iron, silver) is carried out. In terms of continuing to study antimicrobial properties of nanosilver an experimental substance of silver nanoparticles (AgNP) with the size of 30 nm has been studied.

Materials and Methods

Silver nanoparticles were obtained by means of the chemical reduction method in the aqueous medium according to the original protocol developed in F.D. Ovcharenko Institute of Biocolloidal Chemistry of NAS of Ukraine. They were characterized by size using dynamic light scattering (Zetasizer-3, "Malvern Instruments Ltd", Great Britain) and shape using transmission electron microscopy (JEM-1230, "JEOL", Japan).

The evaluation of the antimicrobial activity of silver nanoparticles was carried out in *in vitro* studies using two methods.

1. The antimicrobial activity of AgNP against test-strains of microorganisms was determined according to "MUC 4.2.1890-04, 2004" by the method of serial dilutions in agar and expressed in the concentration of AgNP per volume unit [2]. The initial concentration of the AgNP solution was 800 μg/ml. The following test-strains were used in this study: *Staphylococcus aureus* MRSA ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 2592, *Bacillus subtilis* ATCC 6633 and strains *Shigella sonnei*, *Salmonella typhimurium* 144 obtained from the collection of the State Research Institute of Biotechnology and Strains. The inoculation doses of test-strains were 10^3, 10^4 and 10^5 CFU/cm^3. The antimicrobial effectiveness of AgNP was studied in the final concentrations of 133.8 μg/ml, 100.38 μg/ml, 66.9μg/ml, and 33.46 μg/ml in the nutrient medium (Mueler-Hinton agar). A sterile water dispersion of AgNP was introduced into a sterile Mueler-Hinton agar cooled to 50°C, then mixed and poured on Petri dishes. Cultivation of microorganisms was carried out in thermostat at the temperature of 37°C for 24 h.

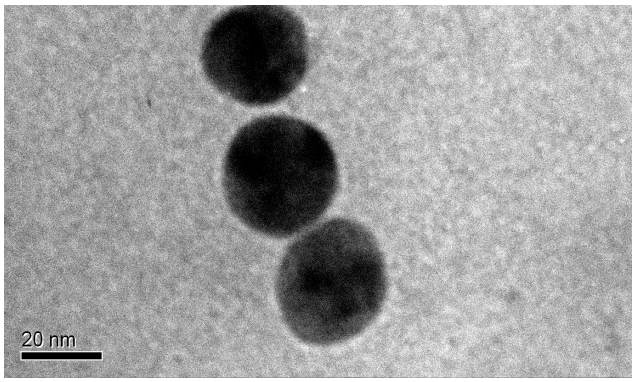


Fig. 1. TEM image of spherical silver nanoparticles of 30 nm in size.

2. The second study was performed on a solid nutrient medium using the method of dosed droplets. Microorganisms involved in the study were multiple drug resistant clinical isolates from wounds, urine, endocervical and faucial scrapings in surgical patients with *Klebsiella ozaenae* 4348, *Citrobacter freundii* 4369, *Escherichia coli* 4358, *Enterobacter aerogenes* 2476, *Proteus mirabilis* 4363, *Staphylococcus aureus* 4312, *Pseudomonas aeruginosa* 283. Microorganisms were inoculated on Mueler-Hinton agar in the concentration of 10^5 and 10^7 CFU/cm^3 to form a bacterial lawn. Suspensions of microorganisms were prepared using 0.9% saline solution. After 30 min of drying of Petri dishes with inoculated bacterial cultures droplets of aqueous dispersions containing nanoparticles with the concentration of 800 μg/ml by metal were applied onto the agar surface. The droplets were 12.5 and 25 μl in volume and contained 10 and 20 μg of silver nanoparticles, respectively. Bacterial cultures were then cultivated in thermostat for 24 h at the temperature of 37°C. The calculation of results was carried out by measuring the diameter of growth inhibition zones. After measurements of growth inhibition zones Petri dishes were stored for 15 days at the room temperature for detecting any secondary growth.

Results and Discussion

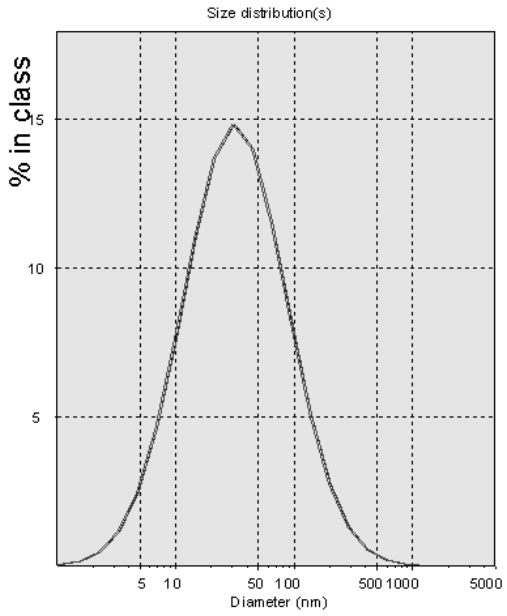
Previous studies proved safety of the AgNP studied, namely genotoxicity, mutagenic action, effect on probiotic bacteria of the gastrointestinal tract [1].

Transmission electron microscopy confirmed that AgNP have a spherical shape (Fig. 1). Results of dynamic light scattering measurements are shown in Fig. 2.

According to the graph it is seen that the size distribution of nanoparticles is monomodal, it means that the colloidal solution has no other fractions. The ZAve parameter shows the average size of nanoparticles. Its average value was 31.8 nm and the absolute error was 0.8 nm.

In Tab. 1 the results of *in vitro* study of the antimicrobial activity of silver nanoparticles against test-strains of microorganisms are given. All strains of microorganisms studied were susceptible to silver nanoparticles. The results obtained indicate a pronounced antimicrobial activity against *Staphylococcus aureus* MRSA ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 2592, *Shigella sonnei*, *Salmonella typhimurium* 144 in the concentration of 33.46 μg/ml. Complete growth inhibition of *Bacillus subtilis* ATCC 6633 was observed at a higher concentration of AgNP – 133.8 μg/ml. This result is important since it is known that *B. subtilis* is a component of the human normal microflora [13]. It is known that bacteria from *Bacillus* genus may develop resistance to silver nitrate and can be used in the process of biological synthesis of silver nanoparticles [10]. The mechanism of appearance of *Bacillus sp.* resistance is uncertain and may be associated with the presence of nitrate reductase enzyme in the bacteria [16].

A pronounced antibacterial activity of AgNPs was also revealed against antibiotic-resistant clinical isolates,



Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	54.4	30.9	1.000	0.001619	13:13:00
2	90.0	54.3	32.1	1.000	0.001599	13:22:35
3	90.0	54.1	32.3	1.000	0.001124	13:32:11
Average		54.3	31.8	1.000		
+/-		0.2	0.8	0.000		

Fig. 2. Dynamic light scattering data of silver nanoparticles. The graph demonstrates distribution of silver nanoparticles by size. The ZAve parameter shows the diameter of nanoparticles , "+/-" – is the absolute error.

Table 1

The antimicrobial activity of 30 nm silver nanoparticles against test-strains of microorganisms

Test-strains	Inoculation dose of test-strains, CFU/cm^3	The final concentration of AgNP drug in the medium, μg/ml by metal				Reference test-strain growth
		133.8	100.38	66.9	33.46	
Staphylococcus aureus MRSA ATCC 43300	10^3	Ø	Ø	Ø	Ø	++++
	10^4	Ø	Ø	Ø	Ø	++++
	10^5	Ø	Ø	Ø	Ø	++++
Pseudomonas aeruginosa ATCC 27853	10^3	Ø	Ø	Ø	Ø	++++
	10^4	Ø	Ø	Ø	Ø	++++
	10^5	Ø	Ø	Ø	Ø	++++
Escherichia coli ATCC 2592	10^3	Ø	Ø	Ø	Ø	++++
	10^4	Ø	Ø	Ø	Ø	++++
	10^5	Ø	Ø	Ø	Ø	++++
Shigella sonnei	10^3	Ø	Ø	Ø	Ø	++++
	10^4	Ø	Ø	Ø	Ø	++++
	10^5	Ø	Ø	Ø	Ø	++++
Salmonella typhimurium 144	10^3	Ø	Ø	Ø	Ø	++++
	10^4	Ø	Ø	Ø	Ø	++++
	10^5	Ø	Ø	Ø	Ø	++++
B. subtilis ATCC 6633	10^3	Ø	Ø	Ø	+++	+++++
	10^4	Ø	Ø	+	+++++	+++++
	10^5	Ø	+	+++	+++++	+++++

Notes: "Ø" – complete inhibition of growth; "++++" – intensive growth; "+++" – weak growth inhibition; "+" – only the growth of single colonies was observed.

and inhibition of bacterial growth was observed in all cases. The results obtained showed that clinical isolates such as *Pseudomonas aeruginosa* 283 and *Klebsiella ozaenae* 4348 which were resistant to the majority of antibiotics, appeared to be susceptible to silver nanoparticles. Diameters of growth inhibition zones for *P. aeruginosa* were the largest among all strains tested. Both gram-negative bacteria from *Enterobacteriaceae* family (*K. ozaenae, E. aerogenes, C. Freundii, E.coli, P. mirabilis*) and gram-positive coccus of *S. aureus* appeared to be susceptible to silver nanoparticles (Tab. 2). Effective concentrations of silver nanoparticles varied from

Table 2

The antimicrobial activity of silver nanoparticles (30 nm) against antibiotic-resistant clinical isolates

Test-strain	Inoculation dose of test-strain, CFU/cm^3	Diameter* of the growth inhibition zone of clinical isolates under the action of AgNP	
		10 μg of AgNP in droplet (by metal)	20 μg of AgNP in droplet (by metal)
Escherichia coli 4358	10^5	10	15
	10^7	11	14
Klebsiella ozaenae 4348	10^5	12	15
	10^7	12	15
Enterobacter aerogenes 2476	10^5	10	13
	10^7	10	12
Proteus mirabilis 4363	10^5	13	17
	10^7	13	17
Citrobacter freundii 4369	10^5	10	15
	10^7	11	14
Pseudomonas aeruginosa 283	10^5	14	20
	10^7	12	20
Staphylococcus aureus 4312	10^5	10	13
	10^7	10	13

Note: * diameters of the growth inhibition zones are expressed in mm.

Table 3

Doses of silver nanoparticles that inhibited growth of antibiotic-resistant strains and expressed in micrograms per surface area of a nutrient medium

Test-strain	Inoculation dose of test-strain, CFU/cm^3	Doses of silver nanoparticles*	
		10 μg of AgNP in droplet (by metal)	20 μg of AgNP in droplet (by metal)
Escherichia coli 4358	10^5	0.13	0.06
	10^7	0.11	0.06
Klebsiella ozaenae 4348	10^5	0.09	0.06
	10^7	0.09	0.06
Enterobacter aerogenes 2476	10^5	0.13	0.08
	10^7	0.13	0.09
Proteus mirabilis 4363	10^5	0.08	0.04
	10^7	0.08	0.04
Citrobacter freundii 4369	10^5	0.13	0.06
	10^7	0.11	0.06
Pseudomonas aeruginosa 283	10^5	0.06	0.03
	10^7	0.09	0.03
Staphylococcus aureus 4312	10^5	0.13	0.08
	10^7	0.13	0.08

Note: * Doses of silver nanoparticles are expressed in μg/mm^2.

0.03 μg/mm^2 to 0.13 μg/mm^2 calculated with reference to the surface area of the nutrient medium (for detailed information see Tab. 3). There was no secondary growth observed in all growth inhibition zones in 15 days of observation.

The mechanism of the antibacterial action of silver nanoparticles is insufficiently studied. The opinion that effect of silver nanoparticles is associated with generation of reactive oxygen species inside the cell is widespread [7, 12, 18, 21]. According to the data [11, 17] a possible mechanism of action of silver nanoparticles includes the complex of the following factors:

- Silver nanoparticles are adsorbed on the surface of the membrane of microorganisms.
- Nanoparticles destroy molecules of lypopolisaccharide and form "sites" of high permeability in the membrane. Silver nanoparticles penetrate inside the cell releasing the Ag^+-ions, which cause the following effects:
  - silver ions interact with cytochromes and block the respiratory chain;
  - silver can also interact with DNA inhibiting its replication.

It has been reported that antimicrobial properties of AgNP depend on size and geometry of particles. According to Kahru A., Dubourguier H.-C. [15] the inhibiting action of nanoparticles against nitrifying bacteria was more pronounced if the size of nanoparticles was less than 5 nm. Pal S. et al. [19] have found that there is dependence between the effect of silver nanoparticles and their geometrical parameters. Thus, AgNP with a triangular shape revealed higher activity than spherical shaped nanoparticles. Researchers explain this regularity by high density of silver atoms in triangular nanoparticles, which along with a high specific surface area provide more active interaction with bacterial cells.

CONCLUSIONS

1. The minimal inhibitory concentration of silver nanoparticles against test-strains of such microorganisms as *Staphylococcus aureus* MRSA ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 2592, *Shigella sonnei*, *Salmonella typhimurium* 144 was 33.46 μg/ml, and it was 133.8 μg/ml against *Bacillus subtilis* ATCC 6633.

2. Silver nanoparticles are active against such antibiotic-resistant clinical isolates as *Klebsiella ozaenae* 4348, *Citrobacter freundii* 4369, *Escherichia coli* 4358, *Enterobacter aerogenes* 2476, *Proteus mirabilis* 4363, *Staphylococcus aureus* 4312, *Pseudomonas aeruginosa* 283. Effective concentrations of silver nanoparticles varied from 0.03 μg/mm^2 to 0.13 μg/mm^2 calculated with reference to the surface area of the nutrient medium.

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АНТИМІКРОБНА АКТИВНІСТЬ НАНОЧАСТИНОК СРІБЛА *IN VITRO*

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***Ключові слова*:** *наночастинки срібла; антимікробна активність; спектр бактерій*

Наночастинки срібла мають великий потенціал у якості антимікробного засобу проти широкого спектра бактерій, включаючи антибіотикорезистентні штами. В даному дослідженні визначені антимікробні властивості синтезованих за оригінальним протоколом наночастинок срібла діаметром 30 нм. В дослідах in vitro із використанням методу серійних розведень у твердому середовищі мінімальна інгібуюча концентрація (МІК) по відношенню до тест-штамів Staphylococcus aureus MRSA ATCC 43300, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 2592, Shigella sonnei, Salmonella typhimurium 144 складала 33,46 мкг/мл. Мінімальна інгібуюча концентрація по відношенню до B. subtilis ATCC 6633 становила 133,8 мкг/мл. Антимікробна активність наночастинок срібла досліджена й на клінічних антибіотикорезистентних ізолятах, що були виділені від хворих хірургічного профілю із ран, сечі, зі скребів з цервікального каналу, зіву: Klebsiella ozaenae 4348, Citrobacter freundii 4369, Escherichia coli 4358, Enterobacter aerogenes 2476, Proteus mirabilis 4363, Staphylococcus aureus 4312 та Pseudomonas aeruginosa 283. Спостерігали повне пригнічення росту досліджуваних клінічних ізолятів при використанні дози наночастинок срібла 10 мг та 20 мг.

ПРОТИВОМИКРОБНАЯ АКТИВНОСТЬ НАНОЧАСТИЦ СЕРЕБРА *IN VITRO*

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***Ключевые слова*:** *наночастицы серебра; противомикробная активность; спектр бактерий*

Наночастицы серебра имеют большой потенциал в качестве противомикробного средства против широкого спектра бактерий, включая антибиотикорезистентные штаммы. В данном исследовании определены противомикробные свойства синтезированных по оригинальному протоколу наночастиц серебра диаметром 30 нм. В опытах in vitro с использованием метода серийных разведений в твердой среде минимальная ингибирующая концентрация (МИК) в отношении тест-штаммов Staphylococcus aureus MRSA ATCC 43300, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 2592, Shigella sonnei, Salmonella typhimurium 144 составляла 33,46 мкг/мл. Минимальная ингибирующая концентрация по отношению к Bacillus subtilis ATCC 6633 составляла 133,8 мкг/мл. Противомикробная активность наночастиц серебра исследована на клинических антибиотикорезистентных изолятах, выделенных из ран, мочи, соскобов из цервикального канала, зева больных хирургического профиля: Klebsiella ozaenae 4348, Citrobacter freundii 4369, Escherichia coli 4358, Enterobacter aerogenes 2476, Proteus mirabilis 4363, Staphylococcus aureus 4312 и Pseudomonas aeruginosa 283. Наблюдалось полное подавление роста исследуемых клинических изолятов при использовании дозы наночастиц серебра 10 мг и 20 мг.