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Antimicrobial activity of colloidal nanosilver 24 ppm *in vitro*

T. P. Popova[1], I. Ignatov[2]*, F. Huether[3], T. Petrova[1]

[1]*University of Forestry, Faculty of Veterinary Medicine, 10 Kl. Ohridski Blvd., Sofia 1756, Bulgaria*
[2]*Scientific Research Center of Medical Biophysics, Sofia, Bulgaria*
[3]*EVODROP AG, Zürich, Switzerland*

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The antimicrobial effect of colloidal nanosilver (AgNPs) at a concentration of 24 ppm against reference strains of *Esherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Clostridium perfringens*, *Candida albicans* and two clinical isolates (*Pseudominas aeruginosa* and *Streptococcus pyogenes*) was examined. The agar diffusion method, determination of the minimum inhibitory concentrations (MIC) and the time of antimicrobial action of AgNPs were used. In the studies performed by the disc-diffusion method, a very good inhibitory effect of AgNPs 24 ppm was reported in all tested microorganisms. The examined Gram-negative bacteria showed higher sensitivity to colloidal nanosilver in comparison with the Gram-positive microorganisms. In the Gram-positive bacteria, the MIC values of the studied AgNPs were higher than in Gram-negative ones. *C. perfringens* with MIC_{50} 0.5 μg/ml showed the highest sensitivity to AgNPs 24 ppm, and *S. aureus* (MIC_{50} 2 μg/ml) - the lowest. AgNPs 30 ppm inactivated all tested Gram-negative bacterial strains within 1 min. In a suspension with a density of 10^6 cells/ml *C. perfringens* died within 24 min. However, individual cells of *S. aureus* remained viable for more than 60 min, and of *S. pyogenes* and *C. albicans* - for more than 120 min. In a suspension with a density of 10^4 cells/ml Gram-positive microorganisms survived for twice as short time - *C. perfringens* up to 15 min, *S. aureus* over 30 min, and *S. pyogenes* and *C. albicans* - for at least 60 min. These results provide hope for the successful use of AgNPs as disinfectant and antiseptic, as well as for topical therapy of infections involving these microbial species.

Keywords: colloidal nanosilver AgNPs, antimicrobial activity

INTRODUCTION

Silver is known as a broad-spectrum antimicrobial agent. Silver ions have long been known to possess a strong and broad spectrum of activities against different bacterial and fungal species. Today, at a time of growing multi-resistance of microorganisms to antimicrobial drugs, interest in silver as an agent with such properties is growing and studies are being conducted in this direction. In this respect, nanotechnologies today are a rapidly growing promising field for development of compounds and materials with antimicrobial properties including such with nanosilver. Colloidal silver consists of nanosized clusters of silver atoms in an aqueous solution. They are of neutral polarity, but their suspension in the water causes a highly negative (interfacial) electrostatic charge (Zeta potential) that causes a mutual repelling action resulting in an almost permanent suspension [1]. Advanced technology also provides electro-colloidal solutions that produce even better results [2]. In the study nanosized Ag colloids were obtained with the addition of urea which produced intermediates AgOCN and Ag_2CO_3 before the formation of silver and obtained silver colloids of average size of 22 nm [3].

Colloidal silver nanoparticles were synthesized using silver nitrate solubilized in the water core of a microemulsion as a source of silver ions, hydrazine hydrate solubilized in the water core of another microemulsion as the reducing agent, dodecane as the oil phase and sodium bis(2-ethylhexyl) sulfosuccinate (AOT) as the surfactant [4]. This nanosilver colloid has low toxicity and high stability, so the colloidal nanoparticles need not be separated from the solution and the silver sol can be directly used as antibacterial agent. Four 1-butyl-3-methylimidazolium halide ionic liquids were synthesized *via* metathesis and anion exchange reactions [5].

By the method of Mosin and Ignatov, colloidal silver can be obtained *via* electrolysis [6]. The synthesis method uses tetra-n-butylammonium bromide in acetonitrile as a liquid medium for electrolysis. The method of Mosin-Ignatov yields colloidal silver with sizes of 2–7 nm in a solution of tetra-n-butylammonium bromide in acetonitrile. The anode is silver, and the cathode is graphite.

The antimicrobial activity of colloidal nanosilver is tested against Gram-positive and Gram-negative microorganisms [5, 7]. According to Liu and Hurt [8], ion release is a cooperative oxidation process requiring both dissolved

* To whom all correspondence should be sent:
E-mail: mbioph@abv.bg

dioxygen and protons. It produces peroxide intermediates. The antibacterial potency, eukaryotic toxicity and environmental release of nanosilver are influenced by the ionic activity associated with the particle suspension. There are studies that support ionic colloidal silver as a broad-spectrum antimicrobial agent against aerobic and anaerobic bacteria, while having a limited and specific spectrum of activity against fungi [9]. Synthesis of stable silver colloids has been successfully achieved by a number of research teams [10-12]. The study has shown a high bactericidal activity of colloidal solutions of nanosilver against strains of *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, as well as fungicidal activity against *Candida albicans* [13].

The purpose of the present work was devoted to testing and evaluation of the antimicrobial action of colloidal nanosilver against Gram-negative and Gram-positive microorganisms from different groups, one of the most common causes of difficult-to-treat infections in humans and animals.

MATERIALS AND METHODS

Device for colloidal nanosilver. Patent Application: PCT/EP2021/054691 [14] 25.02.2021. Title: Control device and method for driving electrodes of at least one electrolysis device for electrochemical production of nanoparticles. Description: The invention relates to the technical field of water treatment. The object of the invention is a control device and a method for driving electrodes of at least one electrolysis device for the electrochemical production of nanoparticles in water according to the generic terms of claims 1 and 10.

Antimicrobial agent. The antimicrobial effect of colloidal silver nanoparticles (AgNPs) at concentrations of 24 ppm was tested.

Control. As a positive control, the broad-spectrum antibiotic thiamphenicol (Nikovet - Sofia) was used, to which the tested microorganisms did not show resistance.

Microorganisms. Pure cultures of 7 pathogenic strains were tested. Five of them were reference: *Esherichia coli* ATCC - 8739, *Salmonella enterica* subsp. *enterica* ATCC 1304, *Staphylococcus aureus* subsp. *aureus* ATCC - 6538, *Clostridium perfringens* ATCC 13124 and *Candida albicans* ATCC 10231. The other two (*Pseudominas aeruginosa* and *Streptococcus pyogenes*) were isolated from cutaneous inflammatory secretions from dogs in the Laboratory of microbiology at the University Clinic at the University of Forestry, Faculty of Veterinary Medicine in Sofia.

Nutrient media. Mueller-Hinton agar and broth (BUL BIO NCIPD - Sofia), Columbia blood agar (Biolab Zrt. H-1141, Budapest Ov. Utra 43) were used, as well as selective media: Endo agar (Antisel - Scharlau Chemie SA, Spain) for *E. coli* and *S. enterica*, Cetrimide agar (Biolab Zrt. H-1141, Budapest Ov. Utr.) for *P. aeruginosa*, Perfringens TSC agar (MkB Test as, Slovak Republic), as well as Zeissler agar (BUL BIO NCIPD - Sofia) for *C. perfringens* and Sabouraud dextrose agar with chloramphenicol (Antisel - Sharlau Chemie SA, Spain) for *C. albicans.*

The cultivation of the microorganisms was carried out at 35-37° C for 18-24 and 72 hours in an anaerobic environment for *C. perfringens* and under aerobic conditions for the other microbial species. The system Anaerob Pack with palladium catalyst - H_2 + CO_2 (BUL BIO NCIPD - Sofia) in Jar was used to create anaerobic conditions. Indic Strip indicator (BUL BIO NCIPD - Sofia) was used to prove the achievement of anaerobiosis.

Preliminary studies of the substance were performed by the *classical agar diffusion method* of Bauer *et al.* [15] and according to Weinstein [16]. Suspensions of 24 h cultures of the test microorganisms were inoculated at a dose of 2×10^6 cells/ml in a volume of 0.1 ml in 9 cm-diameter Petri dishes on Zeissler agar for *C. perfringens* and Mueller-Hinton agar for the other microorganisms, with pH 7.2 - 7.4 and layer thickness of 4 mm. AgNPs 24 ppm and the control antibiotic were administered by instillation of 0.1 ml in 9-mm wells in the agar at concentrations of active substances per well of 30 μg for AgNPs 24 ppm and 30 μg for thiamphenicol. After incubation for 3-4 hours at room temperature for diffusion, the cultures were incubated at 35-37° C for 18-24 and 72 hours. The results were read by measuring the diameters of the inhibitory zones in millimeters, including the diameter of the well to the nearest 1 mm, with a transparent ruler on the outside of the bottom of the plates. According to the three-stage Bauer-Kirby system, an inhibitory effect of AgNPs 30 ppm was observed in areas > 12 mm, and of thiamphenicol – at > 17 mm. The susceptibility of the tested microorganisms to AgNPs 24 ppm was determined as for non-antibiotic preparations such as sulfonamides, namely: resistant (R) - in areas with diameters $\leq$ 12 mm, moderately sensitive - intermediate (I) - in areas in a range of 13 - 16 mm and sensitive (S) at $\geq$ 17 mm. For thiamphenicol the corresponding limits are as follows: R $\leq$ 12 mm, I - 13 - 17 mm and S - $\geq$ 18 mm [17, 18].

Minimum inhibitory concentrations (MICs) were determined by the method of two-fold serial

dilutions in Zeissler agar for *C. perfringens* and Mueller-Hinton agar for the other microorganisms, described by Ericsson and Sherris [19] and NCCLS [17, 18]. Bacterial suspensions were applied at a dose of 10^6 cells/ml. The test preparations of colloidal silver and the control antibiotic were administered in double increasing final concentrations from 1 to 64 μg/ml agar. After incubation at 35-37° C for 18-24 hours, the number of developed colonies was determined. MIC_{50} and MIC_{90} were calculated mathematically based on the number of inhibited colonies on the agar with the respective dilution of the tested compound compared to the colonies on the media with controls without colloidal silver or antibiotic. The range of growth inhibition (D) was defined as the concentration without visible growth.

Determination of the time of antimicrobial action of AgNPs 24 ppm

• A suspension of each of the tested microbial strains at a concentration of 10^5 cells/ml in an amount of 1 ml was added to 9 ml of AgNPs 24 ppm, reaching a final concentration of 10^4 cells/ml.

• A suspension of each of the tested microbial strains at a concentration of 10^7 cells/ml in an amount of 1 ml was added to 9 ml of AgNPs 24 ppm, reaching a final concentration of 10^6 cells/ml.

• The following controls were applied - sterile distilled water (without AgNPs) with the same content of each of the tested microbial strains, as well as 100% AgNPs 24 ppm without microorganisms.

After homogenization for 1 min on a vortex apparatus (Heidolph - Labimex, Bulgaria) and different time intervals for exposure to AgNPs 24 ppm (1 min, 5 min, 15 min, 30 min, 60 min, 120 min and 24 h) cultures were made from each of the samples on Zeissler agar for *C. perfringens* and Mueller-Hinton agar for the other microorganisms, which were cultured at 37° C for 24 - 48 hours under aerobic and anaerobic conditions. After cultivation, the growth of the tested bacteria was reported. The number of colonies developed and the colony forming units (CFU) in 1 ml of the initial suspensions were determined.

All experiments were performed in triplicate.

The statistical processing of the results was performed according to the classical method of Student and Fisher.

RESULTS

In the studies performed by the disc-diffusion method, a very good inhibitory effect of AgNPs 24 ppm (diameters of the inhibitory zones between 20.0 ± 4.7 and 28.7 ± 2.6 mm) was reported in all studied microorganisms. The results are summarized in Table 1. These values were close to those of the control antibiotic, although lower than them. Slightly higher sensitivity to the colloidal nanosilver was shown by the studied Gram-negative bacteria compared to Gram-positive microorganisms ($P>0.05$).

Table 1. Antimicrobial effect of AgNPs 24 ppm against Gram-positive and Gram-negative microorganisms in the agar-gel diffusion method

Microorganisms	Inhibitory zones in mm	
	AgNPs	Thiamphenicol
E. coli	26.7 ± 3.9	36.0 ± 0.8
S. enterica	28.7 ± 2.6	35.3 ± 0.8
P. aeruginosa	25.0 ± 2.4	34.8 ± 0.8
S. aureus	21.7 ± 4.3	25.7 ± 3.3
S. pyogenes	20.0 ± 4.7	26.0 ± 4.5
C. perfringens	22.0 ± 4.0	21.3 ± 0.5
C. albicans	22.7 ± 4.6	28.7 ± 3.7
Total Gram-negative bacteria	25.9 ± 2.0	35.4 ± 0.5
Total Gram-positive bacteria	20.8 ± 2.0	24.3 ± 2.1
Total bacteria	23.3 ± 3.3	29.9 ± 5.7
Total (all microorganisms)	23.1 ± 3.1	29.7 ± 5.3

The highest sensitivity by this method was reported in *S. enterica*, and the lowest - in *S. pyogenes* and *S. aureus*. All tested microorganisms showed high sensitivity to thiamphenicol used as a positive control. The differences in the diameters of the inhibitory zones of the antibiotic and the tested preparation with colloidal silver were statistically significant ($P<0.05$) in the studied Gram-negative bacteria, but not in the Gram-positive ones ($P> 0.05$). The results obtained in determining the minimum inhibitory concentrations are shown in Table 2. They correspond to those obtained in the agar-gel diffusion method. The highest sensitivity to AgNPs showed *C. perfringens* with MIC_{50} of 0.5 μg/ml, and the lowest - *S. aureus* with MIC_{50} 2 μg/ml. For the other microorganisms tested, the MIC_{50} of AgNPs 24 ppm was 1 μg/ml. In Gram-positive bacteria, the values of MIC were higher than those in Gram-negative, but the differences were not significant ($P>0.05$, t-criterion of Student). The values of the control antibiotic were significantly higher - MIC_{50} 4 - 32 μg/ml. As can be seen from the summary data, the bacterial strains tested in this method showed significantly higher sensitivity to AgNPs 24 ppm compared to the control antibiotic ($P<0.01$, t-test of Student).

The results of the experiments performed to determine the sensitivity of the examined Gram-positive and Gram-negative microorganisms to AgNPs 24 ppm, tested at a final concentration of 10^6 cells/ml by the suspension method, are presented in Table 3.

The data showed that AgNPs 24 ppm inactivated all Gram-negative bacterial strains tested within 1 min. Of the Gram-positive microorganisms, the highest sensitivity to colloidal nanosilver is shown by the strict anaerobe *C. perfringens*, which dies within 30 min. However, individual cells of *S. aureus* remain viable for more than 60 min. *S. pyogenes* and *C. albicans* survived longer in the presence of AgNPs 30 ppm. Single cells of these microorganisms remained viable for a minimum of 120 min.

Data from studies performed by the same method, but with a hundred times lower concentration of microbial suspensions (10^4 cells/ml), can be seen in Table 4. All tested Gram-negative bacterial strains died in 1 min. Gram-positive microorganisms at this suspension concentration survived for twice as short a time. *C. perfringens* died within 15 min. However, individual cells of *S. aureus* remained viable for more than 30 min, and *S. pyogenes* and *C. albicans* - for a minimum of 60 min.

Table 2. Minimum inhibitory concentrations of AgNPs 24 ppm against Gram-positive and Gram-negative microorganisms

Microorganisms	AgNPs			Thiamphenicol		
	MIC_{50}	MIC_{90}	D	MIC_{50}	MIC_{50}	D
Escherichia coli	1	2	4	8	16	64
Salmonella enterica	1	2	4	16	32	64
Pseudominas aeruginosa	1	2	4	8	32	64
Staphylococcus aureus	2	4	8	16	32	64
Streptococcus pyogenes	1	2	4	32	64	128
Clostridium perfringens	0.5	1	2	4	8	16
Candida albicans	1	2	4	32	64	128
Total Gram-negative	1.00 ±0.00	2.00 ±0.00	4.00 ±0.00	10.67 ±3.77	26.67 ±7.54	64.00 ±0.00
Total Gram- positive	1.12 ±0.54	2.25 ±1.09	4.50 ±2.18	21.00 ±11.79	42.00 ±2.36	42.00 ±2.36
Total (all microorganisms)	1.07 ±0.42	2.14 ±0.83	4.28 ±1.66	16.57 ±10.57	35.43 ±19.99	84.00 ±47.16

MIC_{50} -50% growth inhibition; MIC_{90} -90% growth inhibition; D – range of full growth inhibition.

Table 3. Antimicrobial effect of AgNPs 24 ppm against Gram-positive and Gram-negative microorganisms in suspensions with a density of 10^6 cells/ml

Microorganisms	Growth of the strains (percent of colony number in comparison with the untreated controls) after different intervals of exposure						
	1 min	5 min	15 min	30 min	60 min	120 min	24 h
E. coli	0	0	0	0	0	0	0
S. enterica	0	0	0	0	0	0	0
P. aeruginosa	0	0	0	0	0	0	0
S. aureus	50	30	20	10	10	0	0
S. pyogenes	65	50	40	25	25	10	0
C. perfringens	35	10	7	0	0	0	0
C. albicans	40	35	22	15	10	6	0
Untreated controls	100	100	100	100	100	100	100

Table 4. Antimicrobial effect of AgNPs 24 ppm against Gram-positive and Gram-negative microorganisms in suspensions with a density of 10^4 cells/ml

Microorganisms	Growth of the strains (percent of colony number in comparison with the untreated controls) after different intervals of exposure						
	1 min	5 min	15 min	30 min	60 min	120 min	24 h
E. coli	0	0	0	0	0	0	0
S. enterica	0	0	0	0	0	0	0
P. aeruginosa	0	0	0	0	0	0	0
S. aureus	25	15	10	5	0	0	0
S. pyogenes	30	25	20	10	5	0	0
C. perfringens	20	5	0	0	0	0	0
C. albicans	35	20	15	10	5	0	0
Untreated control	100	100	100	100	100	100	100

DISCUSSION

The results of the present studies correspond to those of other authors. Petica *et al.* [10] determined a high antibacterial and antifungal activity of some stable colloidal solutions containing up to 35 ppm of Ag. By determination of minimal inhibitorial concentration they found a significant inhibitory effect against Gram-positive and Gram-negative microorganisms such as *S. aureus, E. coli, P. aeruginosa, Acinetobacter* spp. and a fungi mix of *Aspergillus*, *Penicillium* and *Trichoderma* species. Lkhagvajav *et al.* [11] found antimicrobial activity of colloidal silver nanoparticles prepared by the sol-gel method. High inhibitory effect is observed against *E. coli, P. aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae, Bacillus subtilis, S. aureus,* and the fungus *C. albicans* at a very low concentration of 2-4 μg/ml of nanosilver. The authors explained this very high activity with the advantages induced through the sol-gel method since very small nanoparticles can be obtained, which makes them very active against microorganisms. Pokhrel *et al*. [20] also reported antibacterial effect of citrate - functionalized nanosilver on *E. coli.* The results obtained by Petrus *et al.* [2] suggested that colloidal nanosilver exhibits a good bacteriostatic effect but poor bactericidal effect towards food-borne pathogens such as *E. coli*, *Listeria monocytogenes, Salmonella Typhi, Vibrio cholerae, Vibrio parahaemolyticus, Bacillus cereus* and *S. aureus.* However, the research of Batista *et al.* [12] proved the bactericidal effect of colloidal nanosilver and found that the required time to promote the biocide effect is linked to the time-dependent release of Ag^+. Also, this effect is greatly dependent on the presence of Ag^+ rather than the nanoparticles themselves.

The results of Petrus *et al.* [2] support the notion that the antibacterial activity of AgNPs might be due to their adsorption on the bacterial surface, as well as the inhibition of intracellular enzyme activity. Therefore, the remaining Ag ions in the AgNPs solution or dissolved Ag ions are capable of causing a bacteriostatic or even a bactericidal impact. Also, silver antimicrobial property is very high due to their extremely large surface area, which provides better contact with microorganisms.

Mukha *et al.* [13] studied the mechanism of action of nanosilver on microbial cells by a laser scanning confocal microscope using fluorescent label. They found that the first step of the antimicrobial effect on microorganisms is membrane damage and penetration of silver nanoparticles into the cell.

The results of the present study provide hope for the successful use of AgNPs 24 ppm for topical therapy of infections involving the examined bacterial species, as well as for disinfection.

CONCLUSIONS

In the studies performed by the disc-diffusion method, a very good inhibitory effect of AgNPs 24 ppm was reported in all studied microorganisms. The values of the diameters of the inhibitor zones were close to those reported for the control antibiotic, although lower than them. The studied Gram-negative bacteria showed higher sensitivity to colloidal nanosilver in comparison with the Gram-positive microorganisms.

In the Gram-positive bacteria, the MIC values of the studied AgNPs were higher than in Gram-negative ones. *C. perfringens* with MIC_{50} 0.5 μg/ml showed the highest sensitivity to AgNPs 24 ppm, and *S. aureus* (MIC_{50} 2 μg/ml) - the lowest.

The high sensitivity of the obligate anaerobe *C. perfringens* to AgNPs 24 ppm, established by all methods used, is impressive. Higher susceptibility of the studied Gram-negative bacteria, including *P. aeruginosa* - a species that rapidly builds resistance to chemical factors, was found. These results provide hope for the successful use of AgNPs for disinfection, as well as for topical therapy of infections involving these bacterial species. The lowest sensitivity was shown by *C. albicans*, and of the bacteria - by *S. pyogenes*.

The experimental data show that the examined AgNPs 24 ppm possesses well expressed antimicrobial activity and could be successfully used as an antiseptic and for treatment of infections in animals and humans, caused by Gram-positive and Gram-negative bacteria including obligate anaerobes, as well as fungal diseases caused by *Candida albicans*.

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