IN VITRO ANTIMICROBIAL ACTIVITY OF COLLOIDAL NANO SILVER

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Summary

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The antimicrobial effect of colloidal nanosilver (AgNPs) at concentrations of 20 and 30 ppm against reference *Esherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Clostridium perfringens*, *Candida albicans* strains and two clinical isolates (*Pseudominas aeruginosa* and *Streptococcus pyogenes*) was tested. The classical 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 agar diffusion method, a very good inhibitory effect of AgNPs 30 ppm and to a much lesser extent of AgNPs 20 ppm, was reported against all studied microorganisms. The studied Gram-negative bacteria showed higher sensitivity to both preparations compared to the Gram-positive microorganisms (P>0.05). The lowest sensitivity was reported for *S. aureus* and *C. albicans*, and the highest – for *P. aeruginosa* and *S. pyogenes*. The lowest MICs of AgNPs 20 ppm were reported for *P. aeruginosa* and *C. perfringens*. For them, the MIC_{50} was 1 μg/mL, and for the rest – 2 μg/mL. Again, *C. perfringens* showed the highest sensitivity to AgNPs 30 ppm with MIC_{50} 0.5 μg/mL, and the lowest one was that of *S. aureus* with MIC_{50} 2 μg/mL. For the Gram-positive bacteria MIC values were higher than for Gram-negative ones. AgNPs 20 ppm and AgNPs 30 ppm inactivated all bacterial strains tested at final concentrations of 10^3 cells/mL and 10^4 cells/mL within 5 min; only *C. albicans* persisted longer. The tested microorganisms remained viable for significantly longer time in the presence of AgNPs 20 ppm and AgNPs 30 ppm when in suspensions with a density of 10^6 cells/mL. These results are promising for the successful use of AgNPs for disinfection, as well as for topical therapy of infections involving these bacterial species.

Key words: antimicrobial activity, colloidal nanosilver AgNPs, minimum inhibitory concentrations

INTRODUCTION

Today, the problem with the possibilities to choose effective antimicrobials is particularly relevant. Microorganisms are developing resistance to the available antibiotics, and this phenomenon is widespread worldwide posing a serious threat to public health. The adaptability of bacteria at the background of the use of

the same bactericides necessitates new agents with high antimicrobial activity, with different mechanisms of action, to which resistance is neither established nor widespread. The search for new efficient possibilities in this aspect is especially relevant, as well as the expansion of the research on antimicrobial effect and possibilities for application of available preparations with potential in this field. Today, a number of author teams carry out research in order to create various antimicrobial products and test their properties. Nanotechnologies open up perspectives and give hope in this direction. Due to their unique characteristics and capabilities, metal-containing nanoparticles are increasingly included in a wide range of consumer products (Domínguez *et al*., 2020). Recent advances in the creation of drugs based on nanotechnology, open new horizons to combat the resistance of microorganisms to drugs. The use of silver nanoparticles (AgNPs) as a potent antibacterial agent is particularly promising (Dakal *et al*., 2016; Valcheva *et al*., 2000 a,b). Silver is a safe inorganic antibacterial agent that kills many types of pathogenic microorganisms. Its ability to ionise in solutions makes it particularly effective in fighting bacteria, as it dissolves and ionises in water, body fluids, and organic tissues (Domínguez *et al*., 2020; Mohamed *et al*., 2020).

Today, infections caused by multidrug-resistant Gram-negative and Grampositive bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes* and others are a growing global problem. They cause serious intestinal, skin and other soft tissue infections, including pneumonia, endocarditis, septicaemia, nosocomial, postoperative and other diseases,

which are very difficult to treat due to high resistance to antibiotic therapies. *Clostridium* spp. and some serovars of *Salmonella enterica* also show a high incidence of drug resistance. This provokes an urgent need for treatment options with new antimicrobial agents with better bactericidal properties. In this context, interest in colloidal silver is growing. AgNPs are promising for use against infections caused by these and others microorganisms alone or in combination with antibiotics. There is evidence that they can significantly reduce the duration and severity of many bacterial infections (Dakal *et al*., 2016; Pazos-Ortiz *et al*., 2017; Domínguez *et al*., 2020).

In the last decade, silver nanoparticles are used in medicine, pharmaceuticals, chemical, food industry and others due to their unique physical, chemical and biological properties (Balandin *et al*., 2015). Given the current interest in the antimicrobial properties of AgNPs, market applications of products containing silver nanoparticles are expected to increase over the next decade (Rogersa *et al*., 2018). Work is also done to create surfaces with antimicrobial properties (Bryaskova *et al*., 2011). Pazos-Ortiz *et al*. (2017) created a material of silver nanoparticles (AgNPs) embedded in poly-epsilon-caprolactone nanofibres. The antimicrobial activity of materials of this type against various multidrug-resistant Gram-positive and Gram-negative microorganisms showed significant positive correlations related to the dose-dependent effect so they may have significant potential medical applications in infections that are not susceptible to drug therapy.

In the current pandemic, Talebian *et al*. (2020) also recommended greater use of nanotechnology in the development of

highly effective antimicrobial and anti-viral drugs that are suitable not only for air and surface disinfection, but also for personal protective equipment such as facial respirators. Vazquez-Munoz & Lopez-Ribot (2020) pointed out that nanotechnology can be an alternative to reduce the spread of infections, especially in critical areas such as healthcare facilities and public places. Due to the lack of standardised assessment methods, Imani *et al.* (2020) provided a summary of the current state of research aimed at developing antiviral materials and sur-faces, using antimicrobial research as a starting point.

There are, however, some contradic-tory reports on the antimicrobial activity of AgNPs. Related to these features is the purpose of the present work, devoted to testing and evaluation of the antimicrobial action of colloidal nanosilver against Gram-negative and Gram-positive micro-organisms, one of the commonest causes of difficult-to-treat infections in humans and animals.

MATERIALS AND METHODS

Antimicrobial agents

The antimicrobial effect of colloidal silver nanoparticles (AgNPs) at concentrations of 30 ppm and 20 ppm was tested by the method of electrolysis of Mosin & Ignatov (2013) and Ignatov & Mosin (2015). The following ingredients were required: 1) Silver electrode with a purity of 99.99%; 2) Tetra-n-butylammonium bromide (TBAB) – ammonium salt with bromide; 3) Acetonitrile – coloured liquid solution with chemical formula CH_3CN. Tetra-n-butylammonium bromide in acetonitrile was used as a liquid medium for electrolysis. Colloid silver with size of 2–

7 nm was obtained. The anode was silver, and the cathode was from graphite. During the electrolysis a partial dissolving of the silver anode occurred, leading to saturation of the solution with Ag^+. At specified parameters of the electric current and tension over the electrode, the Ag^+ solution concentration was deter-mined by the working time of the electricity source, and the quantity of the water solution.

Control

As a positive control, the broad-spectrum antibiotic thiamphenicol (Nikovet, Sofia, Bulgaria) was used, to which the tested microorganisms showed no resistance.

Microorganisms

Pure cultures of 7 pathogenic strains were tested. Five of them were reference strains from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures: *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 strains (*Pseudominas aeruginosa* and *Streptococcus pyogenes*) were isolated from cutaneous inflammatory secretions from dogs in the Laboratory of microbiology at the University Clinic of the Faculty of Veterinary Medicine at the University of Forestry in Sofia.

Nutrient media

Mueller Hinton agar and broth (BUL BIO NCIPD, Sofia, Bulgaria), Columbia blood agar (Biolab Zrt. Budapest, Hungary) were used, as well as selective media: Endo agar (Antisel - Sharlau Chemie SA, Spain) for *E. coli* and *S. enterica*, Cetrimide agar (Biolab Zrt., Budapest,

Hungary) for *P. aeruginosa*, Perfringens TSC agar (MkB Test as, Slovak Republic), as well as Zeissler agar (BUL BIO NCIPD, Sofia, Bulgaria) 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, Bulgaria) in Jar was used to create anaerobic conditions. Indic Strip indicator (BUL BIO NCIPD, Sofia, Bulgaria) was used to prove the achievement of anaerobiosis.

Preliminary studies of the substances were performed by the classical agar diffusion method of Bauer *et al*. (1966) and according to CLSI (2020). Suspensions of 18−24 h cultures of 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 4 mm. AgNPs 30 ppm and AgNPs 20 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 30 ppm, 20 μg for AgNPs 20 ppm and 30 μg, respectively 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 and AgNPs 20 ppm was observed in areas >12 mm, and of thiamphenicol: at >17 mm. The susceptibility of the tested microorganisms to both AgNPs 30 ppm and AgNPs 20 ppm was determined as for non-antibiotic preparations such as sulfonamides, namely: resistant (R) − in areas with diameters ≤12 mm, moderately sensitive - intermediate (I) − in areas within range 13−16 mm and sensitive (S) at ≥17 mm. For thiamphenicol the corresponding limits were R: ≤12 mm, I: 13−17 mm and S: ≥18 mm (CLSI, 2020).

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 & Sherris (1971) and CLSI (2020). 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

Determination of the time of antimicrobial action of AgNPs 30 ppm and AgNPs 20 ppm was carried as followed:

• A suspension of each of the tested microbial strains in saline solution at a concentration of 10^4 cells/mL in an amount of 1 mL was added to 9 mL of AgNPs 30 ppm as well as of AgNPs 20 ppm, reaching a final concentration of 10^3 cells/mL.

• A suspension of each of the tested microbial strains in saline solution at a concentration of 10^5 cells/mL in an amount of 1 mL was added to 9 mL of AgNPs 30 ppm and respectively of AgNPs 20 ppm, reaching a final concentration of 10^4 cells/mL.

• A suspension of each of the tested microbial strains in saline solution at a concentration of 10^7 cells/mL in an amount of 1 mL was added to 9 mL of AgNPs 30 ppm and respectively of AgNPs 20 ppm, reaching a final concentration of 10^6 cells/mL.

• The following controls were applied – sterile saline solution and distilled water (without AgNPs) with the same content of each of the tested microbial strains, as well as 100% AgNPs 30 ppm, respectively AgNPs 20 ppm, without microorganisms.

After homogenisation for 1 min on a Vortex apparatus (Heidolph, Labimex, Bulgaria) and different time intervals for exposure to AgNPs 30 ppm as well as AgNPs 20 ppm (5 min, 10 min, 15 min, 20 min, 30 min, 60 min , 90 min, 120 min, 150 min, 3 h, 4 h, 5 h 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.

Statistial analysis

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

RESULTS

In the studies performed by the disk-diffusion method, a very good inhibitory effect of AgNPs 30 ppm (diameters of the inhibitory zones between 14.5 ± 0.5 and 17 ± 1.7 mm) was reported in all studied microorganisms. They also showed sensitivity to AgNPs 20 ppm, but to a much lesser extent. The differences in the mean diameters of the non-growth zones at the two tested concentrations were significant (P<0.01). The summarised results are presented in Table 1.

Slightly higher sensitivity (P>0.05) to both silver preparations was shown by the studied Gram-negative bacteria compared to Gram-positive microorganisms. The lowest sensitivity by this method was reported in *S. aureus* and *C. albicans*, and the highest – in *P. aeruginosa* and *S. pyogenes*. All tested microorganisms showed high sensitivity to thiamphenicol used as a positive control, even the tested strain of *C. albicans*. The differences in inhibitory zones diameters of all strains between the antibiotic and the two tested preparations with colloidal silver were statistically significant (P<0.01).

The results obtained from determining the minimum inhibitory concentrations (Table 2) corresponded to those received in the agar-gel diffusion method. The lowest MICs of AgNPs 20 ppm were reported in *P. aeruginosa* and *C. perfringens*. For them, the MIC_{50} was 1 μg/mL, and for the others − 2 μg/mL.

Table 1. Antimicrobial effect of AgNPs 20 ppm and AgNPs 30 ppm against Gram-positive and Gram-negative microorganisms in the agar-gel diffusion method

Microorganisms	Inhibitory zones (mm)		
	AgNPs 20 ppm	AgNPs 30 ppm	Thiamphenicol
Escherichia coli	13.75 ± 1.48	15.00 ± 1.58	24.25 ± 3.30
Salmonella enterica	13.00 ± 0.70	15.50 ± 2.06	19.50 ± 2.95
Pseudominas aeruginosa	13.75 ± 1.92	17.00 ± 1.73	24.25 ± 3.49
Staphylococcus aureus	12.75 ± 1.92	14.50 ± 0.50	21.25 ± 3.56
Streptococcus pyogenes	13.50 ± 1.50	16.25 ± 1.64	18.50 ± 2.69
Clostridium perfringens	12.75 ± 0.83	15.75 ± 1.48	23.75 ± 5.40
Candida albicans	13.50 ± 0.50	15.50 ± 2.06	25.00 ± 3.60
Total Gram-negative	13.50 ± 0.35	15.80 ± 0.85	22.67 ± 2.24
Total Gram-positive	13.12 ± 0.37	15.50 ± 0.64	21.11 ± 2.48
All microorganisms	13.28 ± 0.41	15.64 ± 0.75	22.36 ± 2.40

Again *C. perfringens* showed the highest sensitivity to AgNPs 30 ppm with MIC_{50} of 0.5 μg/mL, and the lowest one was demonstrated by *S. aureus* with MIC_{50} 2 μg/mL. For the other microorganisms tested, the MIC_{50} of AgNPs 30 ppm was 1 μg/mL. In Gram-positive bacteria, the MIC values were higher than those in Gram-negative ones, but the differences were not significant (P>0.05). The values of the control antibiotic were significantly higher (MIC_{50} 4–32 μg/mL). As shown by the summary data, the bacterial strains tested in this method showed significantly higher sensitivity to AgNPs 30 ppm and AgNPs 20 ppm compared to the control antibiotic (P <0.01).

AgNPs 20 ppm and AgNPs 30 ppm inactivated all bacterial strains tested in a suspension with concentration of 10^3 cells/mL within 5 min. Only *C. albicans* endured longer in the presence of AgNPs 30 ppm. Single cells of this fungus survived for at least 5 min, and under the influence of AgNPs 20 ppm – for 30 min. The results from the studies performed in the same method, but with a tenfold higher concentration of the microbial suspensions (10^4 cells/mL), were quite similar to those at the lower tested concentration. In this experimental setting both tested preparations – AgNPs 20 ppm and AgNPs 30 ppm – inactivated all studied bacterial strains within 5 min. However, *C. albicans* remained viable for a longer time – in the presence of AgNPs 30 ppm single cells survived for 5 minutes and under the influence of 20 ppm AgNPs – for 3 hours.

The tested Gram-positive and Gram-negative microorganisms remained viable for significantly longer time in the presence of AgNPs 20 ppm and AgNPs 30 ppm in suspensions with a density of 10^6 cells/mL (Fig. 1 and 2). To inactivate them, a significantly longer exposure of the tested products was required. Under the action of AgNPs 30 ppm, *C. perfringens* (up to 60 min) and *S. pyogenes* (up to 90 min) were inactivated the fastest, and viable cells of *C. albicans* persisted for the longest time – more than 5 hours. Under the influence of AgNPs 20 ppm, the survival time of the tested microorganisms in suspension with a final concentration of 10^6 cells/mL was longer.

Table 2. Minimum inhibitory concentrations of AgNPs 20 ppm and AgNPs 30 ppm against Gram-positive and Gram-negative microorganisms

Minimum inhibitory concentrations (µg/mL)

Microorganisms	AgNPs 20 ppm			AgNPs 30 ppm			Thiamphenicol		
	MIC_{50}	MIC_{90}	R	MIC_{50}	MIC_{90}	R	MIC_{50}	MIC_{90}	R
Escherichia coli	2	4	8	1	2	4	8	16	64
Salmonella enterica	2	4	8	1	2	4	16	32	64
Pseudominas aeruginosa	1	2	4	1	2	4	8	32	64
Staphylococcus aureus	2	4	8	2	4	8	16	32	64
Streptococcus pyogenes	2	4	8	1	2	4	32	64	128
Clostridium perfringens	1	2	4	0.5	1	2	4	8	16
Candida albicans	2	4	8	1	2	4	32	64	128
Total Gram-negative	2.67 ±0.47	3.33 ±0.94	6.67 ±1.88	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	1.75 ±0.43	3.50 ±0.87	7.00 ±1.73	1.12 ±0.54	2.25 ±1.09	4.50 ±2.18	21.00 ±11.79	42.00 ±2.36	42.00 ±2.36
All microorganisms	1.71 ±0.45	3.43 ±0.09	6.86 ±1.81	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; R – range of full growth inhibition.
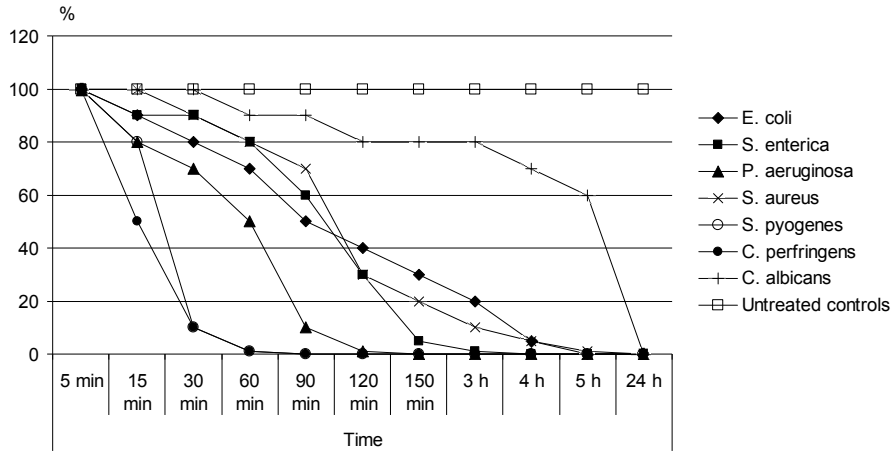
Fig. 1. Antimicrobial effect of AgNPs 20 ppm against Gram-positive and Gram-negative microorganisms in suspensions with a density 10^6 cells/mL, presented as percentage of colonies compared to the untreated control.
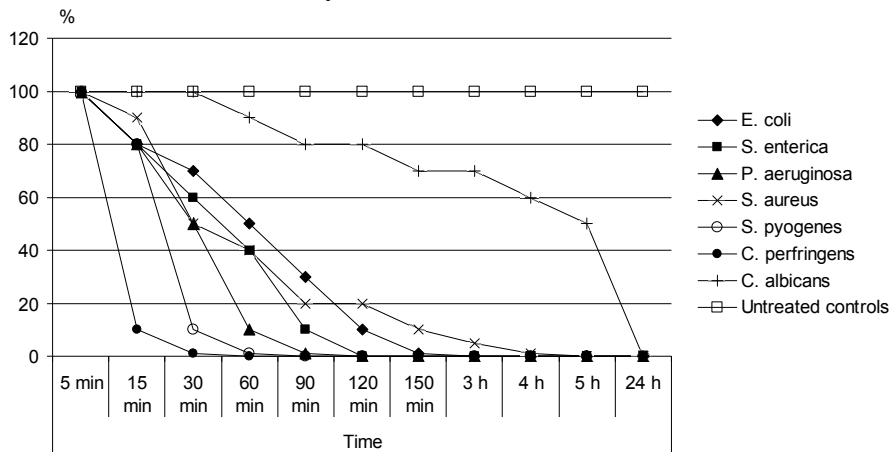
Fig. 2. Antimicrobial effect of AgNPs 30 ppm against Gram-positive and Gram-negative microorganisms in suspensions with a density 10^6 cells/mL, presented as percentage of colonies compared to the untreated control.

Viable *S. pyogenes* and *C. perfringens* cells were preserved for the shortest intervals (60 min), as well as *P. aeruginosa* − for 120 min. Among the bacteria, *S. aureus* survived over the longest period − single cells remained viable even for 5 hours. *C. albicans* showed the highest resistance – more than 5 hours.

DISCUSSION

Colloidal nanosilver is a suspension of submicroscopic silver particles that causes inactivation of the enzymes responsible for respiration, reproduction and metabolism of the treated microorganisms. One of the main characteristics

of the action of silver is its oligodynamic effect (strong microbicidal action of silver ions in water at a very low concentration – 1 ppm). In the ionic monoatomic state (Ag^+) silver is soluble in aqueous medium and shows a strong affinity for sulfhydryl groups and protein residues in cell membranes (Domínguez *et al.*, 2020).

Current studies of the AgNPs antimicrobial activity showed a higher sensitivity of the studied Gram-negative bacteria, including *P. aeruginosa* – a species that rapidly builds resistance to chemical factors. Also, it is necessary to emphasise the particularly high sensitivity of the strict anaerobe *C. perfringens*, established by all methods used in the study. These results give hope for the successful application of AgNPs for disinfection, as well as for topical therapy of infections involving these bacteria. Pazos-Ortiz *et al.* (2017) have also established better antimicrobial effects of AgNPs on Gram-negative bacteria than on Gram-positive strains as bacterial inhibition is directly related to the concentration of AgNPs. Gram-negative bacteria have a cell wall composed of an inner thin peptidoglycan layer and an outer layer of liposaccharides. The small thickness of their cell wall increases the sensitivity of these bacteria to silver ions. Our results are in accordance with these data, obtained by the agar-gel diffusion method. However, the specific response of each bacterium depends on its metabolic characteristics. In Gram-positive bacteria, the cell wall consists of a negatively charged peptidoglycan layer, whose amount is higher than that in Gram-negative bacteria. The lower sensitivity of Gram-positive microorganisms to silver ions can be explained by the fact that their cell wall is much thicker than that of Gram-negative bacteria and contains

teichoic acids, which limit the absorption of silver nanoparticles (Feng *et al.*, 2000; Rai *et al.*, 2012; Dakal *et al.*, 2016; Dominguez *et al.*, 2020).

The present results obtained by the agar-gel diffusion method are in accordance with those of Balandin *et al.* (2015). Their experiments showed that colloidal solutions of silver nanoparticles uniformly inhibited the growth of both Gram-negative and Gram-positive bacteria. The MICs found for *E. herbicola, P. fluorescens* and *B. subtilis* ranged from 0.03 to 0.04 g/L. Pazos-Ortiz *et al.* (2017) reported an established lower effect in the agar-gel diffusion method of nanofibres impregnated with different concentrations of AgNPs relative to Gram-positive bacteria compared to Gram-negative ones. Our results are in compliance with theirs. They also considered that this effect was related to differences in cell wall composition, as the peptidoglycan layer was thicker in *S. aureus* and the silver diffusion therefore reduced. The data of Concepcion *et al.* (2007) are different. Using the disk agar-gel diffusion test, they found out that colloidal silver at 30 ppm was active against *S. aureus*, *S. epidermidis* and *Bacillus subtilis*, but not against *E. coli*.

Our results in studying the antimicrobial effect of AgNPs are consistent with those of Domínguez *et al.* (2020), who reported that colloidal silver had bactericidal activity against Gram-negative and Gram-positive bacteria with MIC_{90} values between 4 and 8 mg/L. These data suggest that colloidal silver may be an effective treatment for infections. Other authors (Shrivastava *et al.*, 2007; Petica *et al.*, 2008; Sintubin *et al.*, 2011) found even more significant differences in the MIC values of colloidal solutions of nanosilver for *S. aureus* and Gram-

negative bacteria, such as *E. coli* and *P. aeruginosa,* compared to our results. They suggested that the process of attachment of the silver nanoparticles to the cell wall surface and penetration through the cell became difficult due to the higher amount of peptidoglycan in the cell walls of Gram-positive bacteria. According to Wheelis (2008), *S. aureus*, whose cells spatially formed clusters, were less sensitive to colloidal nanosilver due to difficulty in penetrating most of the cells which were inaccessible for that reason. This mutual disposition of the cells is possibly a factor in reducing the antibacterial action of colloidal nanosilver and increasing the MIC value (Balandin *et al*., 2015). Our research is consistent with these data. In addition, we found that that in suspensions with a low concentration of bacterial cells, the sensitivity of the tested microorganisms to colloidal nanosilver was significantly increased. Thus, the exposure on the surface of the individual cells was much higher and the binding of the colloidal nanosilver, respectively the antibacterial effect, was facilitated and accelerated. Our results agree with the data of Balandin *et al*. (2015) which demonstrated the bactericidal effect of colloidal solutions of silver nanoparticles applied on the basis of food stabilisers, gum arabic and chitosan, against bacterial cultures of microorganisms. They rightfully considered that data on antibacterial activity and minimum inhibitory concentrations of nanosilver can be used to develop products supressing the activity of microorganisms dangerous to food production.

Mohamed *et al*. (2020) also proved silver as a powerful antimicrobial agent against various microorganisms. Once it enters the bacterial cell, it accumulates as silver nanoparticles with a large surface area, causing cell death. They reported diameters of the zones of inhibition ranging from 16 ± 0.4 to 30 ± 0.23 mm. Their results, as well as ours, give grounds for its use as an effective antibacterial agent, which according to authors can be included in dressings for wounds and burns with high effect, as well as applied as a powerful disinfectant for contaminated water. According to Kooti *et al*. (2018) silver had a significant effect on the bacterial cell wall, causing disruption of its integrity, loss of cytoplasmic content and finally lysis of cells. The research of Mohamed *et al*. (2020) confirmed that silver nanoparticles not only adhered to the cell membrane surface but also penetrated the bacterial cell. Subsequently, they interacted with its DNA, denatured proteins, inactivated its enzymes, generated hydrogen peroxide and for these reasons, caused cell death. The available data suggested that the most dramatic antimicrobial effects of AgNPs were associated with the generation of reactive oxygen species and an increase in oxidative stress, having both cytotoxic and genotoxic effects (Dakal *et al*., 2016).

Our results obtained with regard to estimating the time required to reach the threshold of bactericidal activity are consistent with those of Mohamed *et al*. (2020) who reported a rapid bactericidal effect of the tested colloidal nanosilver after 1 min for *E. coli* and after 15 min for *P. aeruginosa* and MRSA and proved that silver nanoparticles had higher activity than silver ions of $AgNO_3$. These authors also found that silver-killed bacteria could act as an effective remedy against live bacteria, by becoming a reservoir for adsorbed silver nanoparticles. According to them, this phenomenon can play an important role in the treatment of wound infections with the help of dressings

containing silver and for disinfection of water with silver.

Dominguez *et al*. (2020) pointed out that at lower and higher concentrations, colloidal silver induced the formation of reactive oxygen species in Gram-negative bacteria and to a much lesser extent in Gram-positive bacteria, which may explain the slower bactericidal activity of colloidal silver against Gram-positive microorganisms, also found by us. Our results are similar to these of Kim *et al*. (2007) and give us reason to support their view that differences in cell structure, cell wall thickness and composition between Gram-negative and Gram-positive bacteria may explain why *E. coli* was significantly inhibited by silver nanoparticles, while *S. aureus* was less sensitive. Obviously, the antimicrobial potential of silver ions is influenced by the thickness and composition of the cell wall of microorganisms and the difference in the organisation of the peptidoglycan layer. Gram-negative bacteria contain lipopolysaccharides in the outer cell membrane, contributing to its structural integrity, but the negative charge of these molecules stimulates the adhesion of silver and makes bacteria more sensitive to it (Dakal *et al*., 2016). Some basic mechanisms of action of silver ions have already been established. They cause destabilisation of the cell membrane by binding to sulfur atoms present in sulfhydryl groups of proteins and enzymes on the surface of the bacterial cell. Also, silver ions induce the production of reactive oxygen species, inhibition of metabolic pathways by binding silver ions to sulfur-containing proteins, as well as interaction with bacterial DNA and RNA and disorders in cell replication, transcription, translation and reproduction, respectively. Damage to cell structures, including cell envelope,

cytoplasmic membrane, DNA, and RNA, impairs protein synthesis and cell division (Pazos-Ortiz *et al*., 2017; Imani *et al*., 2020; Mohamed *et al*., 2020). In our opinion, this was the reason of the particularly high sensitivity of the strict anaerobe *Clostridium perfringens* to the action of colloidal nanosilver, proven in the present studies. We believe that the formation of reactive oxygen species in bacterial cells under the action of silver ions was a major cause for the rapid bactericidal effect of the colloidal silver shown against *C. perfringens*. The obligate anaerobes to which this species belongs, are highly sensitive to reactive oxygen species. They cannot neutralise them because they do not produce catalase and die quickly under their influence. Tested *S. pyogenes*, although a facultative anaerobe, also belongs to the catalase-negative bacteria. It also showed high sensitivity to AgNPs and this confirmed our assumption that the induction of the reactive oxygen species formation by AgNPs was one of the main factors determining their bactericidal activity. According to Wheelis (2008) and Balandin *et al*. (2015) the arrangement of streptococcal cells in the form of chains was also important. This makes them accessible for easy attachment of silver nanoparticles to the surface of their cell walls and for exerted faster and stronger effect. A particularly positive fact was that silver enhanced the antibacterial activity of a number of antibiotics against *E. coli* and *P. aeruginosa in vitro* and in animal models. In addition, silver was shown to help overcoming antibiotic resistance (Domínguez *et al*., 2020)

Given the results of the present studies, we support the opinion of Vazquez-Munoz & Lopez-Ribot (2020), who emphasise that antimicrobial

nanomaterials may reduce the risk of secondary microbial infections in patients with COVID-19 because they inhibit bacteria and fungi, which can pollute healthcare facilities. Also, nanomaterials show a wide range of activity and they can increase the antimicrobial potency of some drugs, therefore can be used to prevent or treat secondary infections, especially those with multidrug-resistant profiles. Due to its bactericidal properties, silver is a practical antimicrobial agent that will be used for years to come (Mohamed *et al*., 2020).

CONCLUSION

In the studies performed by all methods, a very good inhibitory effect of AgNPs 30 ppm was reported in relation to all studied microorganisms. The effect of AgNPs 20 ppm was significantly smaller. The studied Gram-negative microorganisms showed slightly higher sensitivity to both preparations than the Gram-positive ones. The highest sensitivity was demonstrated by *P. aeruginosa, S. pyogenes* and *C. perfringens* and the lowest − by *S. aureus* and *C. albicans*. AgNPs 20 ppm and AgNPs 30 ppm inactivated all tested bacterial strains at final concentrations of 10^3 cells/mL and 10^4 cells/mL within 5 min. Only *C. albicans* withstood longer. In suspensions with a density of 10^6 cells/mL, the studied microorganisms proved to be much more resistant to the action of coloid nanosilver.

The high sensitivity of the obligate anaerobe *C. perfringens* to AgNPs 20 ppm and AgNPs 30 ppm, established by all methods used, was impressive. Higher susceptibility of the studied Gram-negative bacteria, including *P. aeruginosa* − a species that rapidly builds resistance to chemical factors, was found out. These results seem promising with respect to the successful use of AgNPs for disinfection, as well as for topical therapy of infections involving these bacterial species. Gram-positive bacteria showed higher resistance to AgNPs 20 ppm and AgNPs 30 ppm by all applied methods. The lowest sensitivity was shown by *C. albicans*, and among bacteria − by *S. aureus*.

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