Praca oryginalna *Original paper*

Antimicrobial properties of gold, silver, copper and platinum nanoparticles against selected microorganisms isolated from cases of mastitis in cattle

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Received 01.04.2014 *Accepted 08.05.2014*

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Summary

The study evaluated the antimicrobial properties of commercial preparations containing nanoparticles of silver, gold, copper and platinum against Escherichia coli, Streptococcus uberis, Staphylococcus aureus, Candida albicans and Candida krusei isolated from cases of mastitis in cattle. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the preparations in relation to the isolated microbes were determined. The highest growth-inhibiting activity against the pathogens was noted in the preparations containing nanoparticles of silver and copper, whereas the preparation containing gold nanoparticles had a significantly weaker effect. Platinum nanoparticles at the concentrations applied did not exhibit biocidal activity towards the microorganisms analysed. After 30 minutes the antimicrobial activity of the silver nanoparticles, at concentrations of both 50 and 25 ppm, resulted in the complete elimination of viable cells of the microbes isolated from cases of mastitis. The preparation containing copper nanoparticles exhibited biocidal activity only at a concentration of 50 ppm.

Keywords: Silver, copper, gold, platinum, nanoparticles, antimicrobial effect, mastitis

Mastitis is one of the most frequent and most costly diseases occurring in dairy cattle (6, 7). Economic losses are mainly related to decreased production and lower milk quality, as well as veterinary costs. Among the 150 microbial species involved in the etiology of *mastitis*, infectious and environmental agents are distinguished (2). The infectious pathogens *Staphylococcus aureus* and *Streptococcus agalactiae*, as well as the less common *Corynebacterium bovis* and *Mycoplasma bovis*, are capable of surviving in the mammary gland and can cause inflammation, usually manifested as an increase in the somatic cell count in milk from the infected quarters. Environmental microorganisms, on the other hand, are described as pathogens that exploit the opportunity to induce a mammary gland infection and do not adapt to survive in the host organism. Because this group of microorganisms cannot be completely eliminated from the environment where dairy cattle are raised, the mammary gland is continually exposed to them. This group includes *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Enterococcus* spp., and *Escherichia coli* (11). Also included among environmental pathogens are fungi of the genus *Candida* sp. (24). This complex etiology causes substantial difficulties in selecting effective methods for the prevention and treatment of *mastitis*. Despite numerous negative aspects of antibiotic therapy, this group of drugs is still the basis of successful treatment. A widespread significant increase in antibiotic resistance in microorganisms causing *mastitis* necessitates the search for alternative solutions.

Recent years have seen increasing interest in nanotechnology and its products, nanoparticles, i.e. structures of which at least one dimension does not exceed 100 nm. They have found application in many fields, including medicine, veterinary medicine, and pharmacology (3, 14, 22). A breakthrough in nanotechnology was the development of a physical method for obtaining nanoparticles of silver, gold, and copper. Such fragmentation of nanoparticles increases their biocidal properties in contact with microorganisms. The biological applications of nanomaterials result from their special structure and exceptionally large

active surface, which is of crucial importance in their effect on the cells of microorganisms. Owing to the increased surface area of nanoparticles in relation to their mass, the cell membrane becomes covered with a vast number of nanoparticles, resulting in damage to and deformation of external cell structures (16). Moreover, when nanoparticles bind with thiol groups of amino acids they can cause changes in the conformation of proteins essential for cell life, block centres of enzyme activity, and impede ion exchange with the environment (9, 20). Nanoparticles have also been shown to induce disturbances in the electrochemical potentials of the cell membrane in microorganisms, which can cause significant disruptions in processes taking place on the boundary between the cell and its environment (13). Furthermore, they have been observed to increase the activity of genes responsible for maintaining the cell's redox balance, which is manifested in the death of bacteria and fungi (17). The size of nanoparticles is a decisive factor in fighting microorganisms. A high degree of fragmentation of nanoparticles, within a range of 3 to 10 nm, has been shown to increase their antimicrobial effectiveness (5). The structure of nanoparticles also shapes their biocidal properties. A study by Pal et al. (18) demonstrated that triangular nanoparticles were more effective towards *E. coli* than spheres or rods. Most nanoparticles have a colloid form in which they form the dispersed phase, and the dispersion medium is water (8). These unique properties of nanoparticles of noble metals, in combination with their stability in the environment in which they act, chemical neutrality, and resistance to UV radiation, offer favourable opportunities for their use in the prevention and treatment of many bacterial and fungal diseases.

In view of the antimicrobial properties of nanoparticles, the aim of the study was to determine the antimicrobial properties of nanocolloids of silver, gold, copper and platinum towards selected pathogenic agents isolated from cases of *mastitis* in cattle.

Material and methods

The study was carried out with the use of commercial colloid preparations containing nanoparticles of silver, gold and copper at a concentration of 50 ppm and platinum at a concentration of 20 ppm (Nano-Tech Polska). The antimicrobial properties of the preparations were evaluated in relation to strains from our own collection: *E. coli* (4 strains), *Streptococcus uberis* (4), *Staphylococcus aureus* (3), *C. albicans* (3) and *C. krusei* (3), isolated from clinical cases of *mastitis* in cows.

In the first stage of the experiment the Minimal Inhibitory Concentration (MIC) of the preparations was determined for the test microorganisms by the double dilution method in liquid medium in 96-well flat-bottom microplates (Nunc™). To prepare the inoculum, overnight broth cultures of bacteria were suspended in ISO-Sensitest Broth (Oxoid), and *Candida* sp. cultures were suspended in Yeast Nitrogen Base (Sigma-Aldrich) to a density of $OD_{660} = 1$. The suspensions were finally diluted 1: 50 in growth medium (2×10^4 cells/ml). Next, 100 µl of medium containing nanoparticles was applied to the plates, at concentrations from 25 to 0.39 ppm for gold, silver and copper and from 10 to 0.31 ppm for platinum. Inocula of the microorganisms tested were added in the amount of 100 µl to each dilution. The control consisted of homologous suspensions of the microorganisms in the amount of 200 µl. The microplates with bacterial inocula were incubated at 37°C for 24 h, and those with fungi at 25°C for 48 h. Absorbance was measured in a microplate reader (Bio-Rad 680, Ge) following incubation, at 660 nm. The results obtained were evaluated statistically with Statistica 10.0. The data were analysed by variance analysis with one-way ANOVA to compare differences between absorbance values, and the post-hoc differences were measured by Tukey's test.

In the next stage of the experiment the Minimal Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC) were determined for the preparations containing nanoparticles of silver and copper, which had the highest MIC values against all the microorganisms tested. The test was carried out using nanoparticle concentrations of 50 and 25 ppm. A 10^{-4} dilution was prepared from the inoculum of each of the strains tested, with a density of $OD_{660} = 2$. A 1-ml volume of each inoculum was placed in bacteriological test tubes containing 9 ml of the nanoparticle preparation to be tested. The control was 9 ml of saline solution, pH 7.4. The duration of contact of the nanoparticles and the control sample with the suspension of microorganisms was 30 min. Next, 1 ml of the suspension was plated on Petri dishes and covered with selective media: MacConkey agar (Merck) for *E. coli*, Baird-Parker agar (Oxoid) for *Stapylococcus aureus*, Edwards agar (Oxoid) for *Streptococcus uberis* and Sabouraund agar (BTL) for *C. albicans* and *C. krusei*. The bacteria were incubated for 24 h at 37°C, and the *Candida* sp. strains for 48 h at 25°C. The analysis was performed in 3 replications, and the results were presented as arithmetic means.

Results and discussion

The results obtained show that the silver and copper nanoparticles had the strongest inhibitory effect on the growth of the microorganisms tested (Tab. 1). The silver nanoparticles inhibited the growth of all the microorganisms at a concentration of 12.5 ppm, and in the case of *Staphylococcus aureus*, *Streptococcus uberis* and *C. crusei* this effect was observed at a concentration of ≥ 6.25 ppm. Cu nanoparticles at a concentration of 25 ppm inhibited the growth of all the microorganisms, whereas at a concentration of 12.5 ppm growth inhibition was noted only in the case of *Staphylococcus aureus* and *C. crusei.* In all of these cases OD values differed statistically significantly ($p \leq 0.05$) from the control values. The effect of the preparation containing gold nanoparticles was weaker; statistically significant differences ($p \leq 0.05$) with respect to the control were observed for *Staphylococcus aureus* and *C. crusei* at a concentration of 12.5 ppm and for *Streptococcus* at a concentration of 25 ppm. The weakest inhibitory effect on the growth of the microorganisms was observed in the aqueous solution of platinum nanoparticles. The absorbance values obtained were similar to the control values and were not statistically significant. The microorganisms most susceptible to the nanopreparations

Tab. 1. The average absorbance (S ± SD) from the microorganisms after application of different concentrations of nanoparticles

	Nanoparticles Au (ppm)			Nanoparticles Ag (ppm)			
	25	12.5	6.25	25	12.5	6.25	3.12
E. coli	0.52 ± 0.12	0.58 ± 0.1	0.61 ± 0.2	0.08* ± 0.01	0.06* ± 0.02	0.45 ± 0.1	0.52 ± 0.1
Control	0.64 ± 0.07			0.48 ± 0.09			
Staph. aureus	0.18* ± 0.09	0.22* ± 0.06	0.39 ± 0.06	0.07* ± 0.08	0.017* ± 0.08	0.28* ± 0.06	0.59 ± 0.08
Control	0.48 ± 0.06			0.58 ± 0.08			
Str. uberis	0.24* ± 0.03	0.34 ± 0.01	0.37 ± 0.02	0.11* ± 0.01	0.13* ± 0.06	0.20* ± 0.03	0.40 ± 0.06
Control	0.38 ± 0.03			0.37 ± 0.02			
C. albicans	0.30 ± 0.08	0.49 ± 0.10	0.52 ± 0.08	0.04* ± 0.05	0.05* ± 0.08	0.46 ± 0.05	0.51 ± 0.06
Control	0.53 ± 0.05			0.48 ± 0.08			
C. crusei	0.28* ± 0.05	0.39* ± 0.02	0.52 ± 0.05	0.09* ± 0.05	0.12* ± 0.10	0.18* ± 0.04	0.41 ± 0.02
Control	0.57 ± 0.08			0.58 ± 0.01			

	Nanoparticles Pt (ppm)			Nanoparticles Cu (ppm)		
	20	10	5	25	12.5	6.25
E. coli	0.50 ± 0.1	0.57 ± 0.08	0.59 ± 0.1	0.29* ± 0.02	0.65 ± 0.06	0.95 ± 0.07
Control	0.61 ± 0.06			0.59 ± 0.20		
Staph. aureus	0.60 ± 0.08	0.64 ± 0.06	0.64 ± 0.10	0.40* ± 0.06	0.46* ± 0.07	0.57 ± 0.05
Control	0.68 ± 0.10			0.61 ± 0.08		
Str. uberis	0.25 ± 0.08	0.31 ± 0.03	0.35 ± 0.04	0.19* ± 0.05	0.28 ± 0.03	0.33 ± 0.10
Control	0.37 ± 0.03			0.33 ± 0.06		
C. albicans	0.39 ± 0.09	0.40 ± 0.05	0.43 ± 0.10	0.32* ± 0.05	0.37 ± 0.01	0.44 ± 0.04
Control	0.46 ± 0.06			0.45 ± 0.03		
C. crusei	0.47 ± 0.10	0.57 ± 0.08	0.67 ± 0.08	0.39* ± 0.06	0.45* ± 0.05	0.66 ± 0.11
Control	0.67 ± 0.05			0.66 ± 0.09		

Explanations: * Significant differences in comparison to control ($p \leq 0.05$); Average values ± SD of triplicate

applied were the bacteria *Staphylococcus aureus* and the yeast *C. crusei*. Both were susceptible to nanoparticles of gold and copper, and the values obtained were statistically significantly higher ($p \leq 0.05$) than the control for the concentration of 12.5 ppm, and in the case of the preparation containing nanoparticles of silver, for the concentration of 6.25 ppm. The highest resistance to the preparations tested was observed in the *E. coli* strains, which exhibited susceptibility only to nanoparticles of silver at concentrations ≥ 12.5 ppm and copper at a concentration of 25 ppm.

Analysis of the bactericidal and fungicidal properties of the preparations evaluated indicates that all of the microorganisms isolated from cases of mastitis exhibited greater susceptibility to nanoparticles of silver than copper. As a result of contact between the suspensions of microorganisms and silver nanoparticles at concentrations of both 50 ppm and 25 ppm, total elimination of the cells of the pathogens was attained after just 30 min. (Tab. 2). In the case of the preparation containing nanoparticles of copper, complete inhibition of growth was attained using a concentration of 50 ppm. When the concentration was 25 ppm a few colonies of *E. coli*, *Streptococcus uberis* and *C. albicans* were observed (Tab. 3).

The high antimicrobial activity of nanocolloids of noble metals is confirmed by results obtained by Ruparelia et al. (21). The authors tested the antibacterial

Tab. 2. Numbers of microorganisms following 30-minute contact with silver nanoparticles in comparison with the control

	Nanoparticles Ag 50 ppm	Nanoparticles Ag 25 ppm	Control
Escherichia coli	0	0	1360
Staphylococcus aureus	0	0	1040
Streptococcus uberis	0	0	800
Candida albicans	0	0	1020
Candida crusei	0	0	980

Tab. 3. Numbers of microorganisms following 30-minute contact with copper nanoparticles in comparison with the control

	Nanoparticles Cu 50 ppm	Nanoparticles Cu 25 ppm	Control
Escherichia coli	0	9	1360
Staphylococcus aureus	0	0	1040
Streptococcus uberis	0	4	800
Candida albicans	0	12	1020
Candida crusei	0	0	980

properties of silver and copper nanoparticles towards *E. coli*, *S. aureus* and *B. subtilis.* As in the present study, silver nanoparticles proved more effective than copper against *E. coli* and *S. aureus*. In the case of *B. subtilis* the reverse was true. Copper nanoparticles were more effective against these bacteria than silver, and both MIC and MBC were substantially lower than the values obtained for *E. coli* and *S. aureus*. According to the authors, one reason *B. subtilis* was more susceptible to the antibacterial activity of the copper nanoparticles is the abundance of amino and carboxyl groups on the surface of the bacterial cell and the high affinity of copper for these groups. Moreover, copper nanoparticles may disrupt biochemical processes within the bacterial cell, and by binding to DNA may prevent it from replicating normally. Other studies also confirm the high antibacterial (1, 20, 23) and antifungal (10, 15, 19) activity of nanoparticles of silver, copper and gold. It is significant that these microorganisms have not yet produced strains that are resistant to the different 'physical' biocidal or biostatic mechanism of action of silver and copper nanoparticles. This may be due to the fact that nanoparticles exert their effect simultaneously against different structures, both on the surface of the cell and inside it, while antibiotics have only specific sites of action. Owing to the broad range of activity of nanoparticles towards bacteria and fungi, it is possible to create a universal therapy and decrease the costs of animal treatment. The unique physicochemical properties of nanoparticles offer possibilities for manifold modifications. Research was recently undertaken which for the first time used technology involving non-mechanical binding of a metal, in the form of nanoparticles, with an organic molecule. This technology was used to bind nanoparticles with classic antibiotics. Studies by Geoprincy et al. (4) and Li et al. (12) confirmed the high therapeutic effectiveness of this type of binding. Neither active substance exhibited antagonism with the other, and the result obtained can be regarded as a synergistic effect involving a significant increase in the size of the growth inhibition zones of microorganisms in comparison to the use of each substance separately. To increase the effectiveness of nanoparticles, they are also bound with surfactants. The synergistic effect of silver nanoparticles and sodium dodecyl sulphate (SDS) is confirmed by results obtained by Panacek et al. (19). When this compound was bound with lipids and proteins of the cell wall of *Candida* sp., it increased the accessibility of silver nanoparticles, thus increasing their cytotoxic effect.

The results of this study suggest that preparations containing nanoparticles of silver or copper will play a significant role in controlling microorganisms living in the environment where animals are kept. The use of substances modified by nanotechnology in preparations used for care and disinfection could significantly help in maintaining a low, acceptable level of environmental microbes responsible for inducing *mastitis* in cows. It is significant that preparations containing biocidal concentrations of nanoparticles, unlike those containing metal ions, do not have a cytotoxic effect on eukaryotic organisms. Thus they can become an important complement to preparations that have been used in the prevention and treatment of *mastitis*.

References

1. *Ahmad T., Wani I. A., Manzoor N., Ahmed J., Asiri A. M.*: Biosynthesis, structural characterization and antimicrobial activity of gold and silver nanoparticles. Colloids and Surfaces B. 2013, 107, 227-234.
2. *Bradley A. J.*: Bovine mastitis: an evolving disease. Vet. J. 2002, 164, 116-128.
3. *Chen X., Schluesener H. J.*: Nanosilver: A nanoproduct in medical application. Toxicol. Lett. 2008, 176, 1-12.
4. *Geoprincy G., Saravanan P., Nagendra Gandhi N., Renganathan S.*: A novel approach for studying the combined antimicrobial effects of silver nanoparticles and antibiotics through agar over layer method and disk diffusion method. Dig. J. of Nanomater. Bios. 2011, 6, 1557-1565.
5. *Guzman M., Dille J., Godet S.*: Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. Nanomed. Nanotechnol. Biol. Med. 2012, 8, 37-45.
6. *Halasa T., Huijps K., Østerås O., Hogeveen H.*: Economic effects of bovine mastitis and mastitis management: A review. Vet. Quart. 2007, 29, 18-31.
7. *Huijps K., Lam T. J. G. M., Hogeveen H.*: Costs of mastitis: facts and perception. J. Dairy Res. 2008, 75, 113-120.
8. *Jain P. K., Huang X., El-Sayed I. H., El-Sayed M. A.*: Noble metals on the nanoscale: optical and photothermal properties and some applications in imaging, sensing, biology, and medicine. Acc. Chem. Res. 2008 41, 1578-1586.
9. *Kim J. S., Kuk E., Yu K. N., Kim J. H., Park S. J., Lee H. J., Kim S. H., Park Y. K., Park Y. H., Hwang C. Y., Kim Y. K., Lee Y. S., Leong D. H., Cho M. H.*: Antimicrobial effects of silver nanoparticles. Nanomed. Nanotechnol. Biol. Med. 2007, 3, 95-101.
10. *Kim K. J., Sung W. S., Suh B. K., Moon S. K., Choi J. S., Kim J. G., Lee D. G.*: Antifungal activity and mode of action of silver nano-particles on Candida albicans. Biometals 2009, 22, 235-242.
11. *Lassa H., Kubiak J., Małkińska-Horodyska M.*: Bakterie najczęściej izolowane z klinicznych postaci mastitis u krów oraz ich wrażliwość na antybiotyki. Życie Wet. 2013, 88, 651-653.
12. *Li P., Li J., Wu C., Wu Q., Li J.*: Synergistic antibacterial effects of β-lactam antibiotic combined with silver nanoparticles. Nanotechnology 2005, 16, 1912--1917.
13. *Li W. R., Xie X. B., Shi Q. S., Zeng H., OU-Yang Y. S., Chen Y. B.*: Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli. Appl. Microbiol. Biotechnol. 2010, 85, 1115-1122.
14. *Marambio-Jones C., Hoek E. M. V.*: A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. J. Nanopart. Res. 2010, 12, 1531-1551.
15. *Mishra A., Tripathy S. K., Yun S. I.*: Bio-synthesis of gold and silver nanoparticles from Candida guilliermondii and their antimicrobial effect against pathogenic bacteria. J. Nanosci. Natotechnol. 2011, 11, 243-248.
16. *Morones J. R., Elechiguerra J. L., Camacho A., Holt K., Kouri J. B., Ramírez J. T., Yacaman M. J.*: The bactericidal effect of silver nanoparticles. Nanotechnology 2005, 60, 69-72.
17. *Nagy A., Harrison A., Sabbani S., Munson Jr R. S., Dutta P. K., Waldman W. J.*: Silver nanoparticles embedded in zeolite membranes: release of silver ions and mechanism of antibacterial action. Int. J. Nanomedicine 2011, 6, 1833-1852.
18. *Pal S., Tak Y. K., Song J. M.:* Does the antibacterial activity of silver nanoparticles depend on the shape of nanoparticle? A study of the gram-negative bacterium Escherichia coli. Appl. Environ. Microbiol. 2007, 73, 1712-1720.
19. *Panáček A., Kolář M., Večeřová R., Prucek R., Soukupová J., Kryštof V., Hamal P., Zbořil R., Kvítek L.*: Antifungal activity of silver nanoparticles against Candida spp. Biomaterials 2009, 30, 6333-6340.
20. *Rai M., Yadav A., Gade A.*: Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 2009, 27, 76-83.
21. *Ruparelia J. P., Chatterjee A. K., Duttagupta S. P., Mukherji S.*: Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomaterialia 2008, 4, 707-716.
22. *Singh M., Singh S., Prasad S., Gambhir I. S.*: Nanotechnology in medicine and antibacterial effect of silver nanoparticles. Dig. J. Nanomater. Bios. 2008, 3, 115-122.
23. *Sondi I., Salopek-Sondi B.*: Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. J. Coll. Interf. Sci. 2004, 275, 177-182.
24. *Wawron W., Bochniarz M., Dąbrowski R.*: Antifungal susceptibility of yeasts isolated from secretion of inflamed mammary glands in cows. Pol. J. Vet. Sci. 2010, 13, 487-490.

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