Evaluation of Anti-biofilm and Antibiotic Synergistic Activities of Silver Nanoparticles Against Some Common Bacterial Pathogens

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Abstract

Introduction: Nowadays silver nanoparticles (AgNPs) are used as antimicrobials due to their well-known biochemical properties. The current study was planned to search the antimicrobial activities of AgNPs alone and in combination with common antibiotics against some field isolates of common bacterial pathogens.

Methods: Standard strains of *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Streptococcus agalactiae*, in addition to 9 field isolates for each strain (totally 40 strains, 10 for each) were used. Macrodilution method for determination of minimum inhibitory concentration (MIC) of AgNPs and antibiotics against isolates was used. Biofilm formation was evaluated by microtiter plates. Disc diffusion method was used for assaying bactericidal activities of antibiotics and their combinations with AgNPs against the isolates.

Results: Mean MICs of AgNPs for *Salmonella serotypes*, *E. coli*, *S. aureus* and *S. agalactiae* were 3.125, 6.25, 6.25 and, 12.5 ug/mL, respectively. *S. aureus* and *S. agalactiae* showed more sensitivity (increase in fold) to examined antibiotics plus AgNPs compared to *Salmonella* serotypes and *E. coli*. The results showed that AgNPs had strong antibacterial and anti-biofilm activities against the examined pathogens. Synergistic and antagonistic effects of AgNPs in combination with tetracyclin, gentamicin, streptomycin, kanamycine, cephalosporin and penicillin were observed in different cases.

Conclusion: AgNPs alone or in combination with antibiotics could potentially be used as effective antibacterial and anti-biofilm agents. However, our results showed that synergistic effects of antibiotics combined with AgNPs cannot be considered as a rule.

Keywords: Silver nanoparticles, Antibiotics, Antagonist, Biofilm, Pathogenic bacteria

Introduction

Because of the worldwide increase in multidrug-resistant bacteria, application of novel bactericides in addition to antibiotics for efficient treatments is required.¹ The bactericidal effectiveness of silver nanoparticles (AgNPs) has led to a new generation of therapeutics with ability to reduce bacterial growth.²

The precise mechanism of AgNPs toxic action is not yet fully defined but both the release of silver ions and nanoparticles (NPs) may be involved in their antibacterial activities.³

Apart from intrinsic antibacterial effects of NPs, it is suggested that NPs enhance antibiotic effectiveness when mixed together to treat human and animal bacterial infections.

The synergy between antibiotics and AgNPs has been successfully tested against antibiotic resistant bacteria previously.³

Biofilms are defined as populations of surface-adherent bacteria embedded in an extracellular polymeric matrix containing proteins and DNA which cover the bacterial cell community. Biofilms are diffusion blockades, slowing down the infiltration of

antimicrobials and are known as main causes of chronic infections in medicine.⁴

Biofilms are able to colonize plastic walls of tubes, such as intravenous catheters or tissues like urogenital tract and wounds.^{4,5} Most of the studies on AgNPs antimicrobial activities performed on standard bacterial strains and reports for such studies on field isolates of pathogenic bacteria are rare. Moreover, we could find rare reports regarding studies on AgNPs effects on *Streptococcus agalactiae*.⁵

In the present work, we investigated whether the biofilm formation of some field isolates of common bacterial pathogens including *S. agalactiae* can be influenced by AgNPs at minimum inhibitory concentrations (MIC) and sub-MICs. We also examined the susceptibility of isolates to some antibiotics in combination with AgNPs.

Methods

Bacterial Isolates

Standard strains of *Escherichia coli* ATCC 25922, *Salmonella enteritidis* RTCC 2465, *Staphylococcus aureus* RTCC 1907, and *S. agalactiae* RTCC 1907, in addition to 9 field isolates of each strain (totally 40 strains, 10 for each) were used. *E. coli* isolates were removed from the dead poultry headed to the Veterinary Clinic of Shahrekord University by regional poultry farms. *S. aureus* and *S. agalactiae* were purified from mastitis cow milk in the author's previous works.^{6,7}

Salmonella serotypes were collected from the animals and preserved in the reservoir at Microbiology Laboratory of Veterinary College. The methods (collection and detection of bacteria) were conducted according to Quinn et al guidelines.⁸

AgNPs and MIC Determination

Colloidal AgNPs with particles ranging between 10 and 20 nanometers (Sigma, CAS Number 7440-22-4) were prepared from Kian Eksir Co. which imported AgNPs as powder and prepared its colloidal form. A dilution of 100 µg/mL was prepared from the stock solution (1000 µg/mL) in nutrient broth (Nb) after sonication. Nb was used for all the antibacterial assays. Tube double serial dilution method was used for determination of MIC of AgNPs against isolates, according to guidelines of the Clinical and Laboratory Standards Institute.⁹ The bacterial cultures (in tryptic soy broth, TSB, tubes) were incubated aerobically at 37°C for 18-24 hours. The turbidity of the cultures was adjusted to 0.5 McFarland (1.5×10^8 CFU/mL) and then diluted in saline solution in order to obtain an inoculum of 5×10^6 CFU/tube. Positive and negative controls were also set up.

The inoculated tubes were aerobically incubated at 37°C, shaking, for about 18 hours. The lowest concentration that inhibited visible growth after incubation was defined as MIC which was confirmed in next step.

Anti-biofilm Assays

Biofilm forming abilities of the isolates were evaluated by microplates as described by Tendolkar et al.¹⁰ In brief, the bacteria were cultivated at 37°C in TSB (Merck, Darmstadt, Germany). The strains were afterward centrifuged at $6000 \times g$ (10 minutes), and the precipitate was resuspended in 5 mL of fresh medium. The optical densities (ODs) were adjusted to an absorbance of 1.00 at 595 nm by spectrophotometer (Jenway, OSA, UK). The cultures were admixed (in ratio of 1:40) in fresh TSB. Then 200 µL of cells was inserted in a single row of a sterile polystyrene microtiter plate. The planktonic cells were aspirated after incubation at 37°C for 24 hours, and the wells were rinsed three times with sterile phosphate-buffered saline (PBS). The plates were air-dried for 60 minutes at 25°C. For biofilm tests, 200 µL of aqueous crystal violet (0.2%) was poured in each well and after 15 minutes the wells were washed with sterile PBS to remove the excess dye. Crystal violet was extracted with 200 µL of an 80:20 (vol/vol) mixture of ethyl alcohol and acetone, and its absorbance was read at 595 nm.

To assay AgNPs effects on biofilm formation of the isolates, microtiter plate wells were inoculated with NPs containing examined isolates in such a way that each well contained 10^5 CFU/well and loaded with different concentrations of AgNPs (1.6 – 25 µg/mL).

The plates were incubated at 37°C, shaking, for 24 hours. Control wells were also considered. Growth was assayed using a microtiter enzyme-linked immunosorbent assay (ELISA) reader (Stat Fax 4200) by monitoring absorbance at 600 nm.

Further process was as above. All biofilm and anti-biofilm assays were performed in triplicate.

Biofilm production was judged according to Stepanovic et al.¹¹ In this manner optical density cut-off value (ODc) was calculated as: mean ODs + 3 × standard deviation (SD) of negative control. Accordingly, biofilm producers were classified as: no production when $\leq \text{ODc}$, weak production as $\text{ODc} < \sim \leq 2 \times \text{ODc}$, moderate production as $2 \times \text{ODc} < \sim \leq 4 \times \text{ODc}$, and strong production as $> 4 \times \text{ODc}$, where “~” demonstrates the mean of sample ODs. All strains were tested for biofilm production and 10 isolates in each species that were strong or medium biofilm producers were chosen to calculate the MIC of AgNPs on biofilm formation. Percentage of biofilm inhibition was calculated as explained by Wei et al,¹² using the equation:

$$(1 - A_{595} \text{ of the test} / A_{595} \text{ of non-treated control}) \times 100.$$

Assays for AgNPs and Antibiotics Combined Effects

Antibiotics were selected from 3 groups having different modes of action, including tetracyclin (tetracyclines), gentamicin, streptomycin, kanamycin (aminoglycoside), cephalosporin, and penicillin (beta lactams).

To assay bactericidal activities of different antibiotics

against the isolates, disc diffusion was exploited on Muller–Hinton agar (MHA) plates. The standard antibiotic discs were purchased from Techno Biolab Co. To determine their combined effects, each antibiotic disc was impregnated with 20 μ L solution (1 MIC for each isolate) of AgNPs. Similar tests were performed with antibiotic discs alone. An isolate colony of each strain was cultured in TSB medium (37°C, overnight). The inocula were then exerted to the plates along with the standard and prepared discs. The inhibition area was then determined. All the tests were done in triplicate.

Fold area changes were determined by measuring the mean surface area of the inhibition zone. Changes were then calculated as $b^2 - a^2 / a^2$, where a and b are inhibition areas for the antibiotic and combination of antibiotic and AgNPs respectively.³

Results

Mean MICs of AgNPs for *S. serotypes*, *E. coli*, *S. aureus* and *S. agalactiae* isolates were 3.125, 6.25, 6.25 and 12.5 μ g/mL, respectively. All strains were selected based upon their abilities to form strong to medium biofilms. Percentages of biofilm formation, inhibiting 40 isolates in the presence of different concentrations of AgNPs are presented in Table 1.

In the present work, using the disc diffusion method, the combined activities of AgNPs and antibiotics (gentamicin,

tetracycline, streptomycin, cephalosporin, kanamycin and penicillin) were studied against 40 isolates of common pathogenic bacteria (*S. serotypes*, *E. coli*, *S. aureus* and *S. agalactiae*) in comparison to antibiotics alone. The diameters of inhibition zones have been noted in Table 2. *S. aureus* and *S. agalactiae* showed more sensitivity (increase in fold) to examined antibiotics plus AgNPs compared to *S. serotypes* and *E. coli*. For the 2 recent bacteria only penicillin and tetracycline plus AgNPs showed fold increasing. Maximum increase in fold area was related to kanamycin and tetracycline (8 and 2.2 respectively) against *S. agalactiae*.

Discussion

In the present work, we evaluated the antibacterial and anti-biofilm formation activity of AgNPs against 2 gram-negative (*Salmonella* serotypes and *E. coli*) and 2 gram-positive (*S. aureus* and *S. agalactiae*) bacteria that are considered important pathogens. The mean MICs of AgNPs recorded for gram-negative (3.125-6.25 μ g/mL) and gram-positive (6.25-12.5 μ g/mL) bacteria were well comparable with other reports regarding examined species.¹³ Thus we could conclude that the AgNPs inhibited growth of the examined isolates, and *E. coli* was more susceptible to AgNPs concentrations compared to *S. agalactiae* (Table 1).

Most of the reports on AgNPs antimicrobial activities are performed on standard bacterial strains; here we examined some field isolates of pathogenic bacteria in addition to their standard counterpart.

In a study by Helmlinger et al,¹⁴ the impact of AgNPs on the biology of prokaryotic and eukaryotic cells was scrutinized. In the mentioned report, it was shown that cells with a higher specific surface area such as platelets faded faster than particles with a smaller specific surface area such as cubes.

It is suggested that the antimicrobial effects of AgNPs may be related to silver ions shedding because bacteria are probably unable to ingest the used AgNPs in contrast

Table 1. MICs (μ g/mL) and Percentages of Biofilm Inhibition by Nanosilver Particles in 40 Field Isolates of Bacterial Pathogens

Bacteria	Mean MICs	AgNPs concentrations ^a		
<i>E. coli</i>	3.125	1.6 (93)	3.125 (95)	6.25 (96)
<i>S. serotypes</i>	6.25	3.1 (92)	6.25 (95)	12.5 (96)
<i>S. aureus</i>	6.25	1.6 (90)	3.125 (92)	6.25 (96)
<i>S. agalactiae</i>	12.5	6.25 (92)	12.5 (94)	25 (92)

^a Numbers in parentheses are mean percentages of biofilm inhibition for different concentrations of silver nanoparticles against 10 isolates of each bacterial species.

Table 2. The Mean Zone of Inhibition (mm) of Different Antibiotics Against *Escherichia coli*, *Salmonella* serotypes, *Staphylococcus aureus* and *Streptococcus agalactiae* in the absence and presence of silver nanoparticles^a

	<i>E. coli</i>			<i>S. serotypes</i>			<i>S. aureus</i>			<i>S. agalactiae</i>		
	Ab	AgNPs	†	Ab	AgNPs	†	Ab	AgNPs	†	Ab	AgNPs	†
Gentamicin	2	2	0	20	20	0	20	25	0.56	20	25	0.56
Streptomycin	3	3	0	10	10	0	17	22	0.67	20	20	0
Tetracycline	10	12	0.44	4	7	2	20	22	0.21	15	20	2.2
Cephalosprine	5	5	0	4	4	0	28	35	1.2	20	30	1.25
Kanamycin	5	5	0	25	25	0	27	27	0	5	15	8
Penicillin	4	4	0	23	27	0.73	30	34	1.1	20	25	0.56

^a Numbers are mean inhibition zones of 10 isolates for each species.

† denotes for fold increases of different antibiotics against examined isolates that were calculated as $(b^2 - a^2)/a^2$, where a and b are the inhibition zones for antibiotic and antibiotic plus AgNPs, respectively.

Ab denotes for antibiotic.

to eukaryotic cells.¹⁴ To confirm the latter idea, Wang et al found that gold nanospheres can attach to the membrane of *Salmonella typhimurium*, but unable to be digested by the bacteria.¹⁵

AgNPs with a concentration range of 1.6-6.25 µg/mL exhibited more than 90% inhibitory effects on biofilm formation of the examined species (Table 1). Generally, the inhibition of the biofilm formation relates to the inhibition of bacterial growth. Studies have shown that chlorhexidin, triclosan and phenolic compounds, among others, may also inhibit biofilm development.¹⁶

Hence, the effective anti-biofilm concentration of AgNPs could be lower than their antibacterial concentration and at the same concentration, higher anti-biofilm activity is expected.

Gurranthan et al also reported AgNPs inhibitory concentrations were somehow low in such a manner that could affect cell viability. Gram-positive bacteria of *S. aureus* and *Streptococcus pneumoniae* treated with 0.7 µg/mL of AgNPs showed 90% reduction in biofilm activity.¹⁷

A recent study on *Staphylococcus epidermidis* showed that anti-biofilm activity of AgNPs was dose- and time-dependent.¹⁸ The anti-biofilm activity of AgNPs against antibiotic resistant bacteria was reported in similar studies.¹⁹

As appeared in Table 2, the results clearly showed that antibacterial activity against gram-positive bacteria (*S. aureus* and *S. agalactiae*) was more when antibiotics plus AgNPs were used rather than when antibiotics were used alone, as evidenced by increase in fold area. This combinatory effect does not seem to be fully successful for examined *Salmonella* serotypes and *E. coli* isolates.

Fayaz et al²⁰ noted that synergistic effect may be a result of conjugative reaction between antibiotic and AgNPs. They showed inhibition of gram-positive bacteria was generally more difficult to be obtained by AgNPs alone, as our results were also in line with their observation.

In the case of isolates of *S. aureus* and *S. agalactiae*, our results were in line with other findings such as a report by Birla et al.³ They reported synergistic activities between antibiotics like vancomycin, gentamicin, streptomycin, ampicillin and kanamycin, and AgNPs against *Pseudomonas aeruginosa*, *S. aureus* and *E. coli*. However for *Salmonella* serotypes and *E. coli* isolates the story was to some extent different (Table 2), and this finding challenged Gurranthan et al¹⁷ suggestion regarding higher toxicity of individual AgNPs or antibiotics rather than combined form against bacterial cells.

De Souza et al reported an antagonistic activity of AgNPs with amoxicillin or oxacillin combinations against a methicillin resistant *S. aureus* strain.²¹

Kazemi et al²² assessed the efficacy of AgNPs in combination with 4 antibiotics against 50 isolates of mastitis-causing *S. aureus*. A table in their report shows when AgNPs were combined with streptomycin and erythromycin, 33 and

16 (out of 46) isolates (respectively) showed antagonistic activities for the combinations, however we could not find any reference to this antagonistic activity in their paper.

Hari et al observed that in some cases, the combination of antibiotics with AgNPs brought down the inhibitory effect of antibiotics. In their report, AgNPs were recommended with and without antibiotics for controlling the pathogens.²³ Taking together, our results confirmed that synergistic effects of antibiotics combined with AgNPs cannot be considered as a rule.

Since AgNPs may have different shapes and sizes, and be modified with different coatings, this may lead to differences in their effects in combination with different antibiotics.

There are also other reports that show antibacterial and anti-biofilm activities of antibiotics combined with AgNPs against *P. aeruginosa*, *Shigella flexneri*, *S. aureus*, and *S. pneumoniae*. An enhanced activity for ampicillin and vancomycin against both gram-negative and gram-positive bacteria has also been reported.¹⁷

However, reports regarding AgNPs effects on *S. agalactiae* are rare⁵ and as appeared in Table 1, we can conclude that this species is more resistant to antimicrobial activities of AgNPs compared to our other examined isolates.

In conclusion, our results demonstrated that AgNPs shows promising anti-biofilm activity against field isolates of *S. serotypes*, *E. coli*, *S. aureus* and *S. agalactiae*. These particles can also compete with commercial antibiotics used for the treatment of bacterial infections and sometimes are even better.

Regarding examined *S. aureus* and *S. agalactiae* isolates, a possible combination of tetracyclin, gentamicin, streptomycin, kanamycin, cephalosporin and penicillin with AgNPs showed enhanced antimicrobial effects and was concluded as synergism. However, synergistic effects of combination of antibiotics and AgNPs cannot be considered as a rule. Further research on animal models is required.

Conclusion

AgNPs alone or in combination with antibiotics could potentially be used as an effective antibacterial and anti-biofilm agent. Synergistic effects of antibiotics combined with AgNPs cannot be considered as a rule.

Ethical Approval

The study protocol was approved by Local Ethics Committee of Veterinary College, Shahrekord University.

Competing Interests

Authors declare that they have no potential conflict of interests.

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