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Original article

In vitro bactericidal and imipenem synergistic effect of nano-silver against multiple drug-resistant *Pseudomonas aeruginosa*

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ABSTRACT

Background: Pseudomonas aeruginosa (*P. aeruginosa*) is an aerobic gram-negative pathogen. It induces decline of lung functions and increase mortality rate. It has a high antimicrobial resistance rate limiting number of antibiotics which can be used.
Objectives: Evaluation of bactericidal and antibiotic synergistic effect of Nano-silver (Ag-NP) against Multiple drug-resistant *P.aeruginosa* (MDR *P. aeruginosa*).
Material and methods: P.aeruginosa were isolated from clinical specimens. MicroScan WalkAway-96SI System was used for laboratory Identification, antimicrobial susceptibility. Minimal inhibitory concentration (MIC) was determined by a microdilution method. Time kill assay determined by incubation MRSA with different concentrations of Ag-NP (0, 50, 100, and 200 μg/ml) in a shaking incubator at 37 °C for 24 h. Growth curves of bacterial cell cultures were attained through repeated measures of the optical density (O.D.) at 600 nm. The disc diffusion method was used to evaluate synergistic of Ag-NP with antibiotics.
Results: The MIC value of Ag-NPs against MDR *P. aeruginosa* was in at of 5 μg/ml. The bacterial growths of cells treated with 1.25, 2.5, 5 and 10 μg/ml Ag-NPs were inhibited. After 4 h, almost all treated bacterial cells were dead. All of the combinations showed significant synergistic effect (P-value 0.003), and the result showed the highest synergism at concentrations of Imipenem at MIC (32 μg/ml) and 16 μg/ml.
Conclusion: Nano-Silver has high therapeutic activity against MDR *P. aeruginosa*, it can be suggested as an alternative or adjuvant with antibiotics for MDR *P. aeruginosa* treatment. Further studies are required for understand synergistic effect of Ag-NP combining, and assessment of its safety.

1. Introduction

Pseudomonas aeruginosa is a widespread nosocomial pathogen (Fowler and Nancy, 2014; Breidenstein et al., 2011), can cause to cause dangerous opportunistic infections among immunocompromised patients, for example, chronic lung infections in cystic fibrosis patients (Winstanley et al., 2016). *P. aeruginosa* has been classified as a superbug because of its extraordinary adaptability, intrinsic resistance to antibiotics and ease of acquiring microbial resistance, which also contribute to its multidrug resistance and pan-drug resistance to existing antibiotics (Livermore, 2002; Oliver et al., 2015).

The rapid incidence and spread of MDR *P. aeruginosa* strains leads it a high morbidity and mortality of infections caused by MDR *P. aeruginosa* (Fowler and Nancy, 2014). Clinically isolated strain, specially, have witnessed a serious situation. Therefore, there is an urgent need to develop novel antimicrobial compounds or combinations with potent antibacterial activity against clinically isolated MDR *P. aeruginosa*.

Among the range of materials whose antimicrobial property is being investigated, AgNP appear as a promising new antibacterial agent that could be helpful to confront this and other drug-resistant bacteria. Different studies have established the bactericidal effect of Ag-NP in Gram negative and Gram-positive bacteria, but the bactericidal mechanism of this compound has not been clearly elucidated. Morones et al. (Yoon et al., 2008) defined the antibacterial activity of silver nanoparticles in four types of Gram-negative bacteria: *Escherichia coli, Vibrio cholera, P.aeruginosa, and Salmonella tiphy* and suggested that Ag-Np attach to the surface of the cell membrane and disturb its function, penetrate bacteria, and release silver ions (Morones et al., 2005).

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Silver nanoparticles showed antibacterial activity against some drug-resistant bacteria (Inoue et al., 2010; Birla et al., 2009). In addition, several studies regarding the synergistic activity of Ag-Np in combination with other compounds have been reported: a combination of amoxicillin and Ag-NP showed greater bactericidal efficiency towards *E.coli* than when they were applied separately (Li et al., 2005), and interactions between Ag-NP and polymyxin B showed synergistic effects for Gram negative bacteria (Ruden et al., 2009).

Carbapenem such as imipenem and meropenem play key role treatment of *P. aeruginosa* infections. Lately, a problem of carbapenem resistance has loomed with emergence of Carbapenem resistant *P. aeruginosa* (Maltezou, 2009; Lister et al., 2009). Treatment of imipenem-resistant infection is very difficult, due to imipenem resistance genes are located on transferable genetic elements such as plasmid. These infections are associated with high mortality and morbidity rates (Bebrone, 2007). In the present study, we investigated the antimicrobial activities of Ag-NP alone and in combination with Imipenem against MDR *P. aeruginosa.*

2. Material methods

2.1. Bacterial isolate

Ninety *P. aeruginosa* isolate from clinical specimens at Microbiology laboratory, Department of clinical analysis at Gynecology and Children hospital, Hafr Elbatin, KSA. Mid-stream urine, suction tip, pus and blood specimens were collected aseptically for bacteriological examination. Handling, transporting, and storing of collected samples were made at refrigeration temperature. All samples were inoculated on Blood agar, incubated at 37 °C for overnight, and colonies were processed.

2.2. Antimicrobial testing

The MicroScan WalkAway-96SI System was used in laboratory Identification, antimicrobial susceptibility testing and *P. aeruginosa* detection was performed with Neg Combo Panel Type 32 (Dade Behring®, USA). All procedures were performed according to the manufacturer's instructions.

2.3. Antimicrobial activity of NANO-silver

Nano-silver were obtained from SIGMA ALDRICH, SAINT LOUIS, USA. MIC defined as the lowest concentration of AgNP preparation that prevented bacterial growth. MIC The minimal inhibitory concentration (MIC) was determined by a microdilution method, using LB broth (Sigma–Aldrich) and final inoculum of 105 CFU/ml. Bacteria were incubated with serial twofold dilutions of Ag-NP, and the effect on cell viability was measured after a 24 h period of incubation. Bacterial cell viability was measured by using a colony-forming capacity assay in nutrient agar (Kohanski et al., 2007). All the assays were run in parallel with negative and positive control.

2.4. Time-kill assay

To examine the growth curves of bacterial cells exposed to Ag-NPs, Mueller-Hinton broth with different concentrations of Ag-NPs powder (0, 1.25, 2.5, 5, and 10 μg/ml) was used, and the bacterial cell concentration was adjusted to 10^5 CFU/ml (Salomoni et al., 2017). Each culture was incubated in a shaking incubator at 37 °C for 24 h. Growth curves of bacterial cell cultures were attained through repeated measures of the optical density (O.D.) at 600 nm.

2.5. Synergistic effect of nano-silver with antibiotics

The disc diffusion method was used to evaluate the synergistic of silver nanoparticles with antibiotics. Based on the CLSI standards (Wayne, 2011). By using the spread plate method, the Mueller-Hinton agar plates were inoculated with the turbidity adjusted bacterial suspension, and antibiotic discs (Hi Media Chemicals Pvt. Ltd., Mumbai, India), were placed on plates containing MDR *P. aeruginosa* and was incubated at 37 °C for 24 h after which the inhibition zone diameter of plates containing colloidal silver, MDR *P. aeruginosa,* and antibiotic disc and those containing only the antibiotic disc and *P. aeruginosa* organisms were measured, and zone of inhibition (ZOI) was measured by subtracting the disc diameter from the total inhibition zone diameter. The synergistic effect was quantified by the equation $(B - A)/A \times 100$, where A and B are the ZOI for antibiotic and antibiotic + silver nanoparticles, respectively.

2.6. Statistical analysis

All experiments were conducted in triplicate to validate the producibility of the experiments. Statistical analysis was carried out by one-way ANOVA at a P-value of 0.05 by Microsoft Excel software (Microsoft, USA).

3. Results

3.1. Bacterial strains

Of ninety *P. aeruginosa* isolates, 15 were MDR *P. aeruginosa.* Multiple drug-resistant *P. aeruginosa* was defined as isolate showed resistance to three or more of the following eight sentinel antimicrobial agents: amikacin; aztreonam; cefepime; ceftazidime; ciprofloxacin; colistin; imipenem; and piperacillin/tazobactam.

3.2. Antimicrobial activity of nano-silver

Cell viability assay was used to assess the bactericidal effect of Ag-NP, multiple-drug resistant *P. aeruginosa* was subjected to two-fold Ag-NP serial dilutions for 24 h. Nano-Silver affected bacterial cellular viability in a dose-dependent manner. Bacterial cell viability was measured by using a colony-forming capacity assay. MDR *P. aeruginosa* was inhibited at concentrations over 5 μg/ml at 10^5 CFU where no visible bacterial growth in agar plate.

3.3. Time-kill assay

Growth curves of *P. aeruginosa* treated with Ag-NPs showed that Ag-NPs can inhibit the growth and reproduction of bacterial cells, Fig. 1. The bacterial growths of *P. aeruginosa* treated with 0, 1.25 2.5, 5 and 10 μg/ml Ag-NPs were inhibited. After 4 h, almost all treated bacterial cells were dead. The bacterial growth of the cells treated with 1.25 and 2.5 μg/ml Ag-NPs were also lower than that of cells in the control group.

3.4. Synergistic effect of nano-silver with imipenem

Nano-Silver showed a significant synergistic effect when it combined with imipenem P-vale 0.003, Table 1. Also, the synergistic effects of Ag-NP were investigated with Imipenem against MDR *P. aeruginosa* using the disc diffusion method, and the effects evaluated by determination of the synergism percentage. The result showed the highest synergism at concentrations of Imipenem at (32 μg/ml) and 16 μg/ml, Fig. 2.

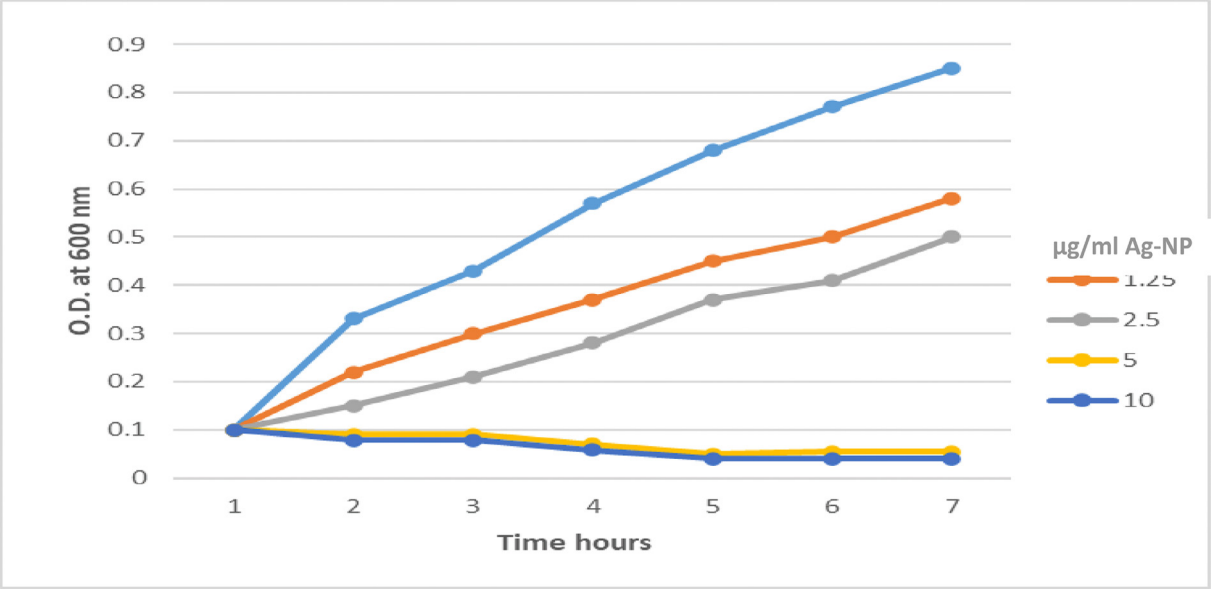

Fig. 1. Growth curve of MDR *P. aeruginosa* with different concentrations of Nano-silver.

Table 1
Statistical analysis of inhibition zones of different imipenem concentration combined with Ag-NP at different combinations.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1066.8	2	533.4	9.212435233	0.003764567	3.885294
Within Groups	694.8	12	57.9			
Total	1761.6	14				

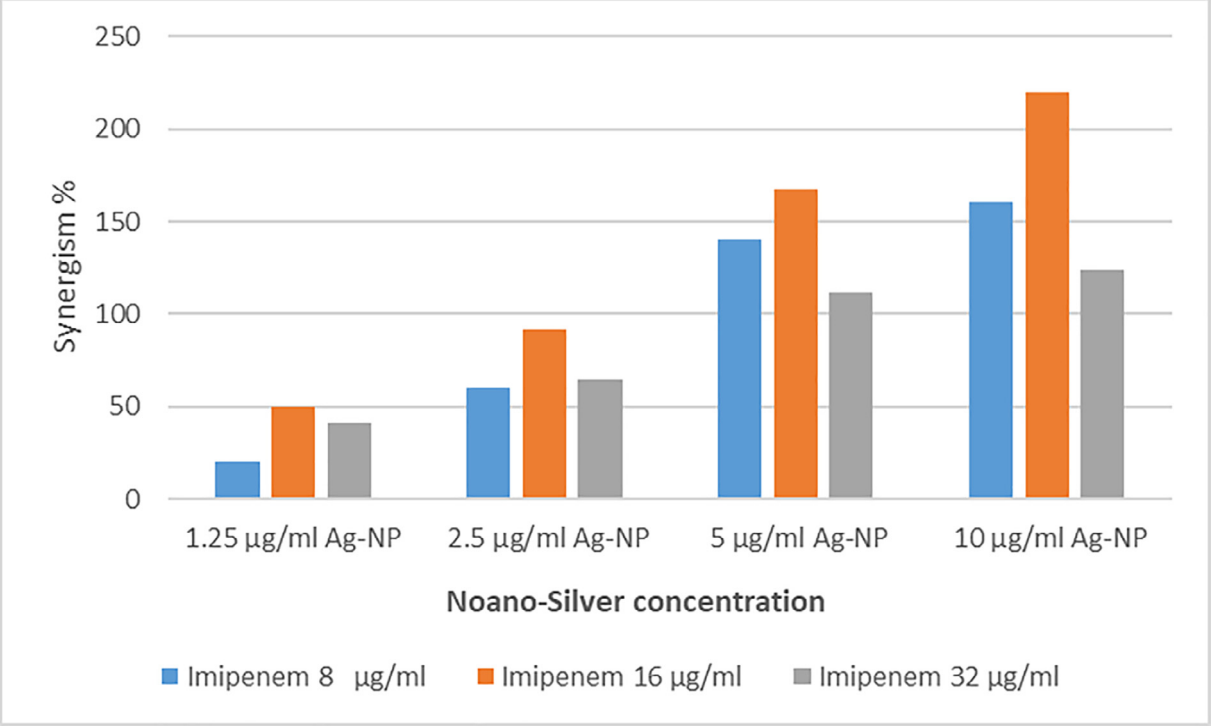

Fig. 2. Synergistic effect of combination of Ag-NP with Imipenem at different concentrations for them.

4. Discussion

The increasing trend of antimicrobial resistance alarms about the dilemma that could be faced when treating these infections. The surveillance results showed a high incidence of MDR *P. aeruginosa* in our hospitals.

Many studies evaluated the antibacterial activity of Ag-NP (Chaloupka et al., 2010; Gade et al., 2010). Nano-Silver reported

antibiofilm and antibacterial effect against multidrug-resistant bacteria, this helps in solving the most serious problem to worldwide public health (Ghotaslou et al., 2017; Ansari et al., 2011). Here, we evaluated the antibacterial activity of Ag-NP against MDR *P. aeruginosa*, the results showed increases in the antibacterial activity of Ag-NP directly with the increasing of Ag-NP, Fig. 1. The results obtained by Tiwari et al. showed that treating bacterial cells by Ag-NP caused protein leakage from the bacterial cell along with increasing of Ag-Np concentration and led to cell death (Tiwari et al., 2008). The current study reported complete susceptibility of MDR *P. aeruginosa* over MIC (5 μg/ml), Fig. 1. This proves the works of other authors that colloidal silver is a powerful antimicrobial agent that can destroy over 650 microorganisms at a very low concentration without having any deleterious effect on the body tissues (Caufield et al., 2000). These findings indicate that the antibacterial activity of 2.5 μg/mL of Ag-NPs could slightly inhibit bacterial growth yet not enough to outpace the speed of reproduction of the bacterial cells. Many researches have been performed studying the effect of Ag-NPs on pathogenic microorganisms.

Our results showed that the combination of Ag-NP with Imipenem significantly increased its activity (P-value 0.003), Table 1, and indicate the synergistic effect of Ag-NP. The result showed that Ag-NP influenced Imipenem activity at different concentrations with the highest effect at combinations of Imipenem/AgNP (32/10 μg/ml) and (16/5 μg/ml), Fig. 2. A similar result was obtained by Shaimaa et al., the combination of chemical and biological Ag-NP with Imipenem significantly increased the antimicrobial activity of Imipenem (Hasson et al., 2019). The results reported by Fayaz et al. (2010) and Rai et al. (2012) on *P. aeruginosa* were somewhat similar to our findings.

The probable mechanism involved in the synergistic effect of antibiotics with Ag-NPs could be the formation of complexes between the antibiotics and Ag-NPs; through the bonding of the antibiotic's active and functional groups like hydroxyl and amino groups to the large surface area of Ag-NPs by chelating (Fayaz et al., 2010; Oni et al., 2002), Silver Nanoparticles antibacterial mechanism works by inhibiting oxygen metabolism, which finally kills the microbes in a very short time (Shahverdi et al., 2007).

In the current study combinations of Ag-NPs and Imipenem showed enhanced antimicrobial activity of Imipenem against MDR P. aeruginosa and overcome resistance problem. These are encouraging results, as it may be possible to achieve an effective antimicrobial effect at lower antibiotic concentrations against multiple-drug resistant bacteria. Further studies are required to understand the synergistic effect of nanosilver combinations and assess the safety and efficacy of new antibiotics Ag-NPs combinations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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