

Anti-bactericidal and anti-biofilm activities of silver nanoparticles against multidrug-resistant Gram-negative bacilli isolated from burn wound infections

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ABSTRACT

Background: The emergence and spread of multidrug-resistant Gram-negative bacilli in burn wound infections related to biofilm formation, which lend to challenge in treatment with conventional antibiotics and prompting to search for novel antimicrobial agents to control the infections. Silver nanoparticles (AgNPs) have wide spectrum biological properties with different mechanisms of action and less toxicity towards human cells.

Objective: The goal of this study was to evaluate the anti-bacterial and anti-biofilm activities of AgNPs alone and in combination with aminoglycoside (Amikacin) and β -lactam (Ampicillin) antibiotics against multidrug resistant Gram-negative bacilli (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) isolated from burn wound infections.

Type of the study: Cross-sectional study.

Methods: 70 clinical isolates of GNB tested for susceptibility tests by disk diffusion method against 10 antibiotics. The minimum inhibitory concentrations (MICs) of AgNPs and antibiotics were carried out according to the standard broth microdilution method, while synergistic interactions were evaluated by time kill-kinetic assays. Calgary method was applied for anti-biofilm activity.

Results: *Pseudomonas aeruginosa* represented the majority of GNB isolated from burn wound infections 34 (48.5 %) followed by *Klebsiella pneumoniae* 21 (30 %) and *Escherichia coli* 15 (21.5 %). Silver nanoparticles showed remarkable antibacterial activity against GNB that isolated from burn wound infections with the MICs between 25- 75

$\mu\text{g/ml}$. Aztreonam, amikacin and cefepime were the most effective antimicrobial drug against GNB isolates. Synergistic bactericidal effects were observed in two-drug combinations of AgNPs with broad-spectrum aminoglycoside (Amikacin) and β -lactam (Ampicillin) antibiotics against multidrug resistant GNB. In addition, AgNP alone or in combination with ampicillin inhibited biofilm activity about 60 % - 75 % of GNB, while combination of AgNPs with amikacin exhibited a powerful anti-biofilm activity and inhibition biofilm formation by 75% to 80%.

Conclusion: The results confirmed a synergistic bactericidal effects and significant enhancing of anti-biofilm activity of AgNPs in combination with antibiotics (amikacin and ampicillin) against multidrug resistant GNB isolated from burn wound infections. These data suggest that AgNPs could be applied as nanodrug for treatment of burn wound infections.

Keywords: Burn wound infections, Silver nanoparticles, Anti-bactericidal, Anti-biofilm, Gram-negative bacilli

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Burn wound infections are still considered a serious public health problem and the most significant cause of morbidity and mortality in developing countries(1). Approximately 55-85% of mortality amongst thermally injured and burn patients attributable to complications of infections(2). Mortality rates related to contamination of burn with bacteria and fungi are over 70%, and Gram-negative bacilli (GNB) have the major mortality rate among all burn patients with bacterial infection. Gram-negative bacilli are equipped with a group of virulence factors that enable it to colonization on burn and facilitate tissue invasion leading to speedy transition into the bloodstream causes bacteraemia and sepsis(3). The most prevalent microorganisms that have been related to burn wound infections involve aerobic bacteria "*Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Proteus*" and anaerobic bacteria "*Bacteroides fragilis*, *Fusobacterium Spp.*, *Propionibacterium Spp.*, and *Peptostreptococcus*" and fungi "*Candida Spp.*, *Zygomycotic*, and *Aspergillus niger*" (4). Colonization of microorganisms on burn or wounds

initially starts as biofilm formation, most bacteria isolated from burn wound infections, especially Gram-negative bacteria are able to produce biofilms within 10 - 72 hours. Development of biofilm is major virulence factors that responsible for profoundly resistant to antimicrobial treatment and to the mechanism of the host immune system that leads to increased risk of contamination and systemic infection. The increase of multidrug-resistant bacterial in burn wound infections related to biofilm formation, making treatment difficult with traditional antibiotics prompting to search for new strategies to solve this problem(5). Nanoparticles are currently viewed as a reasonable other option to antibiotics and appear to have a high potential to tackle the issue of the development of bacterial multidrug resistance, specifically Gram-negative bacteria isolated from skin and bone infections(6). Silver nanoparticles (AgNPs) have pulled in much consideration in the medical field. AgNPs have been observed to be active against numerous human pathogenic microorganisms "Bacteria, fungi, parasites, and viruses". Additionally, AgNPs possess many biological activities including anti-inflammatory, anti-cancer, and anti-angiogenic activities. This wide spectrum biological properties of AgNPs due

to different mechanisms of action and less toxicity towards human cells(7). The present study was aimed to evaluate the anti-bacterial and anti-biofilm activities of AgNPs alone and in combination with broad-spectrum aminoglycoside (Amikacin) and β -lactam (Ampicillin) antibiotics against multidrug resistant Gram-negative bacilli "*Pseudomonas aeruginosa*, *Escherichia coli*, *klebsiellapneumoniae*" isolated from burn wound infections.

Methods

Sample collection and bacterial identification :The samples were collected from 70 patients who were admitted in the burns center at Al-Yarmouk hospital and specialist burns hospital in Baghdad medical city for the period between October 2016 and April 2017. Sterile wound swabs were used to collect the samples from open burn wounds. The swabs specimens were transported inside 60 minutes to the laboratory for direct smear and culture to identification of pathogenic bacteria. Two swabs collected from each patient, the first swab was stained by Gram stain and the second swab was placed into liquid media (Brain heart infusion broth) for overnight incubation than about 100 μ l sub-cultured onto Blood agar and MacConkey agar then incubation aerobically at 37 °C for 24 h. The identification of bacterial growth confirmed by cultural characteristics and biochemical tests using the API 20 E kit "BioMérieux, Marcy L'Etoile, France".

Antibiotics and silver nanoparticles (AgNPs): Antibiotics tested in this study were purchased from Bioanalyse (Turkey) and Oxoid (UK): "Aztreonam, Gentamicin, Amikacin, Ciprofloxacin, Ceftazidim Piperacillin-tazobactam, Amoxicillin-clavulanate". Silver nanoparticles were obtained from Hongwu International Group Ltd (China) with the following specifications: appearance "grey black powder", morphology "Spherical", particle size (20nm), purity (99.99%), apparent density (0.97g/ml), and tap density (2.16g/ml) (Figure 1.)

Antibiotics Susceptibility Test (AST). : Antimicrobial sensitivity test against Gram-negative bacilli isolates were performed according to modifying Kirby-Bauer's disk diffusion method recommended by Clinical Laboratory Standards Institute (CLSI) guidelines. Three to five similar appearance colonies of Gram-negative bacilli including "*Pseudomonas aeruginosa*, *Escherichia coli*, *klebsiellapneumoniae*" selected from overnight incubation on blood agar (Oxoid, UK) at 37°C and colonies were suspended in to sterile plain tubes contains sterile saline (0.9% NaCl) for adjusted inoculum to 0.5 McFarland standard turbidity "approximately cell density 1.5×10^8 CFU/ml". The bacterial suspension plated on Müller-Hinton agar using sterile swab according to streaking method and antibiotic discs placed on the agar plates by sterile forceps. after incubation at optimal temperature (37°C) for 18-24h. Diameter of zone of inhibition sizes around the antibiotic discs measured and documented in millimeter (mm). The final results record as susceptible "S" or resistant "R" as indicated by the criteria organized by the (CLSI) (8).

Determination of minimum inhibitory concentrations (MIC) of antibiotics and AgNPs.

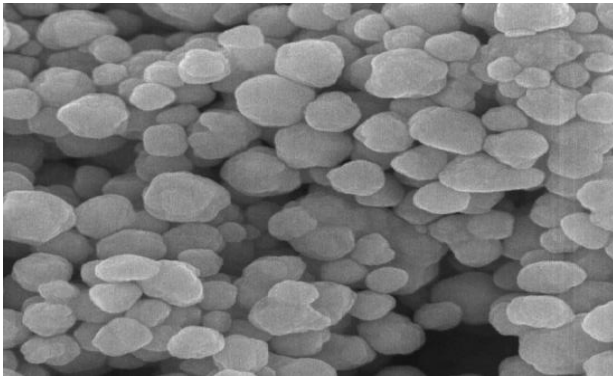
:The minimum inhibitory concentrations of AgNPs and antibiotics were carried out according to guidelines of CLSI standards "Clinical and Laboratory Standards Institute". Gram-negative bacilli strains were grown in Müller-Hinton broth media and inoculum suspensions were prepared using a spectrophotometer (Labtronics, India) with turbidity equal to 0.5 McFarland standard to obtain a final concentration of approximately 1×10^6 . Then, 0.1 ml of inoculum suspensions of bacteria dispensed to each well of a 96-well microtiter plate which contains two-fold serial dilution of antibiotics or AgNPs(9). The microtiter plates were incubated at 37 °C for 18-24h and MIC values were assayed with enzyme-linked immunosorbent assay microtiter reader "Huma Reader-HS, Human GmbH, Wiesbaden, Germany" by checking absorbance at 600 nm. Bacteria with Müller-Hinton broth used as positive control, while sterile water with media used as negative control.

Time kill-kinetic assays: Time kill-kinetic assay was performed according to the National Committee for Clinical Laboratory Standards "NCCLS" guidelines to evaluate the effect of combination between AgNP and selected antibiotics against *Escherichia coli*, *klebsiellapneumoniae*, and *Pseudomonas aeruginosa*. Bacterial cells were grown in fresh medium of Müller-Hinton broth and bacterial suspended to get a final concentration about 1×10^6 CFU/ml. Then, inoculum suspensions add to tubes contains different concentrations of AgNPs or antibiotics. After incubation tubes at 37 °C aliquots of 100 μ l taken from each sample at "0, 2, 4, and 24 h" and planted on agar media to calculate the number of colony forming unit (CFU) and determines synergistic effect(10).

Evaluation of anti-biofilm activity by the tissue culture plate technique (TCPT).

The anti-biofilm formation activity of AgNPs alone or in combination with selected antibiotics were performed by Calgary biofilm method with some modification. This technique depended on colorimetric quantities measurement of the crystal violet. Briefly, Gram negative bacilli strains were grown overnight at 37°C and diluted with lysogeny broth to give a concentration of 1×10^6 CFU/ml. Then, 180 microliters of suspension add to 96-well microtiter flat-bottom polystyrene plate and incubated for 24h at 37°C. AgNPs and combination of drugs were added in different concentrations to each well then plates were incubated for 4 h at room temperature. The microtiter plates washed twice or three times with 200 μ l of phosphate buffered saline and allowed biofilms to fixed in the bottom of well after incubation for one hour. Crystal violet was used to stain biofilms, 200 μ l of 1% crystal violet were add to each well and incubated at room temperature for 45 minutes followed by destained with 95% ethanol for 40 min at 37°C. The absorbance at 595 nm of each well was recorded by microtiter plate reader "Huma Reader-HS, Human GmbH, Wiesbaden, Germany" The percentage of anti-biofilm activity was calculated according to the following equation "[1-(A 595 of cells treated with AgNPs/A 595 of non-treated control cells)] \times 100"(11).

Statistical Analyses: All biological tests in this study were done in triplicate and the experiments repeated at least twice. The data were displayed as mean ± standard deviation (SD). Graphpad PRISM® 6 software “GraphPad Software, Inc., La Jolla, CA, USA” was used to statistics analysis. Student’s t-test was applied to analyzed of P-values. P < 0.05 accepted as statistical significant.



1.Morphology of Silver nanoparticles (20nm) Hongwu International Group Ltd. S* = Significant (P < 0.05)

Results :During the seven-month period of this study, seventy of Gram-negative bacilli isolated from burn wound infections from the patients who registered in the specialized burns hospital in the medical city of Baghdad and burning Center at Yarmouk Hospital. In this study, *Pseudomonas aeruginosa* represented the majority of Gram-negative bacilli isolated 34 (48.5 %) followed by *Klebsiella pneumoniae* 21 (30%) and *Escherichia coli* 15(21.5%) with no statistical significance between species (P > 0.05) as presented in figure 2.

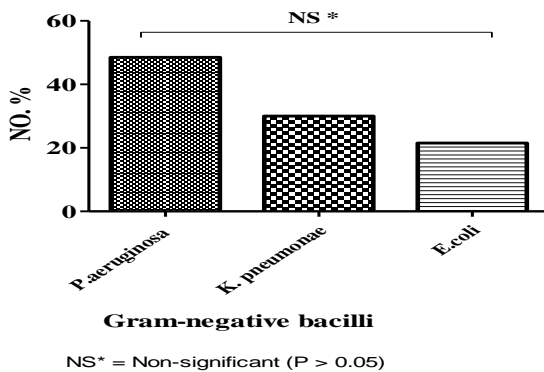


Figure 2. Types of Gram-negative bacilli isolated from burn wound infections.

The results of the current study showed that Gram-negative bacilli were spread significantly in burn wound infections among age groups of 20 -29 years old with significantly different between age groups (P < 0.001). The patients with burn wound infections with age below ten or over fifty years old displayed lower exposure to infection with Gram-negative bacilli strains and mean age of the patients was (26 ± 6) year. Furthermore, female patients showed high

risk to be contaminated with Gram-negative bacilli rather than male patients and the male / female ratio was (0.75/1) as described in the figure-3-.

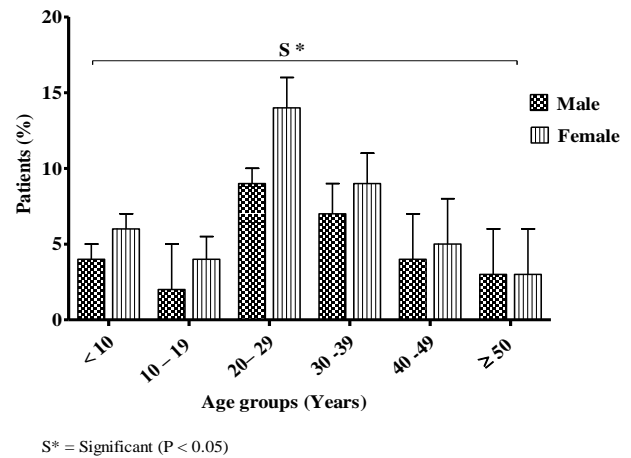


Figure 3. Distribution of the burn patients according to sex and age groups.

Silver nanoparticles used in this study with the of size 20 nm showed antibacterial activity against Gram-negative bacilli that isolated from burn wound infections with the minimum inhibitory concentration (MIC) between 25- 75 µg/ml. *Escherichia coli* was the most sensitive Gram-negative bacilli to the activity of AgNPs with MIC 25 µg/ml, followed by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with MIC 50 µg/ml and 75 µg/ml respectively. Statistical analysis showed significant differences between the strains for respond to AgNPs activity with P value less than 0.5 as shown in figure 4.

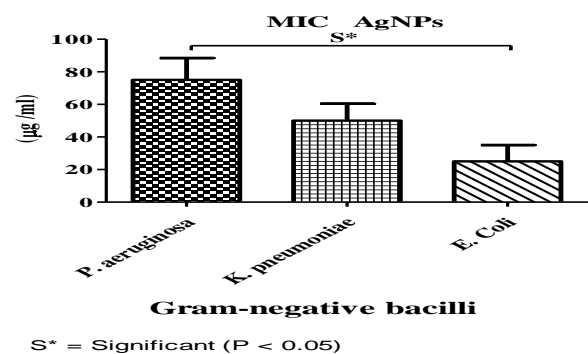


Figure 4. Antibacterial activity of AgNPs against Gram-negative bacilli.

All Gram-negative bacilli strains in this study were tested for antibiotic susceptibility test by modifying Kirby-Bauer’s disk diffusion technique using ten antibiotics that have a different mechanism of action. Multidrug-resistant (MDR) strains of Gram-negative bacilli were significantly isolated from clinical samples and the most bacterial isolates exhibited markedly resistance to ampicillin, followed by amoxicillin- clavulanate and gentamicin. In addition, aztreonam, amikacin and

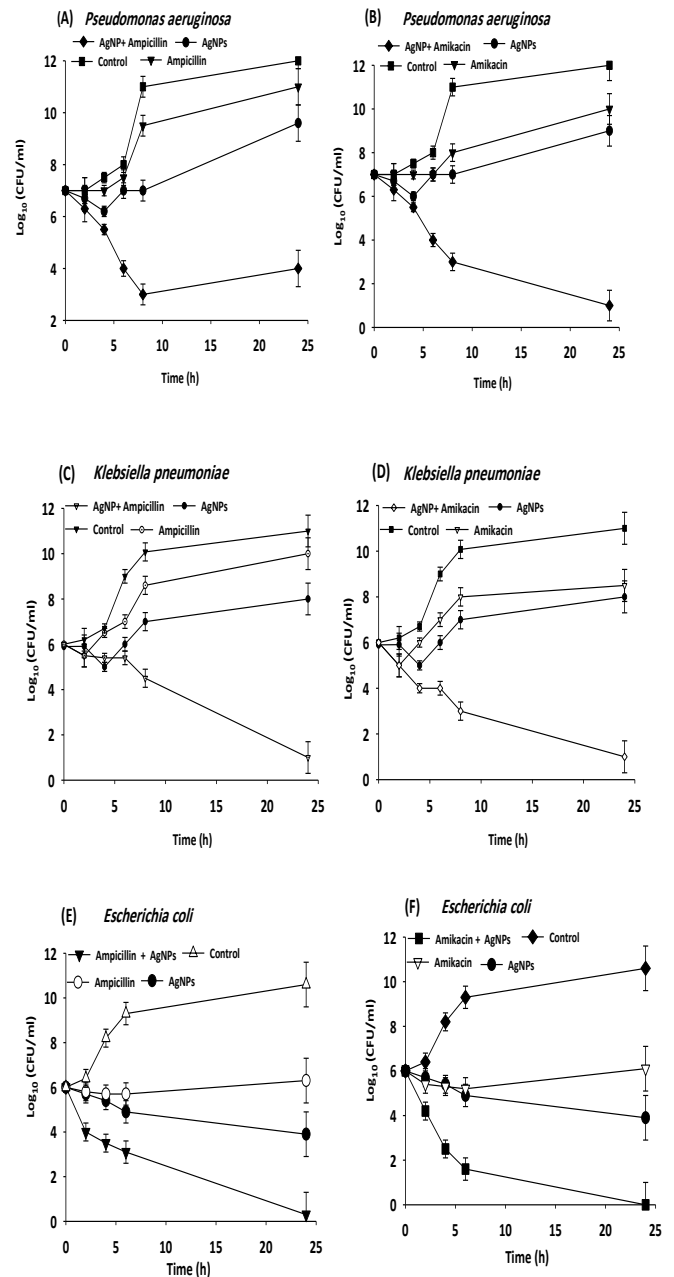
cefepime were the most effective antimicrobial drugs against Gram-negative bacilli isolates. *P. aeruginosa* showed high level of resistance against the most tested antimicrobial agents, especially against ampicillin (97.4%) and amoxicillin- clavulanate (87.8 %) and moderately resistant to gentamicin (61.1 %) and cefotaxime (56.9%). *K. Pneumonia* showed less resistance than *P. aeruginosa*, ceftazidime and cefotaxime were the most active antibiotics against *K. Pneumonia* with (21.3%) and (22.4%) of resistances respectively. In addition, *Escherichia coli* represented the lower resistance against most tested antibiotics, amikacin, cefotaxime, and aztreonam exhibited the minimal rate of resistances against *Escherichia coli* with a percentage of (8.6%), (14.9 %), and (21.3 %) respectively as shown in the table1.

Table 1. Antibiotic susceptibility test of Gram-negative bacilli isolated from burn wound infections

N O .	Antibiotic s	Sym bol	Co nc. (μ g)	<i>P. aeruginos</i>		<i>K. pneumoniae</i>		<i>E. Coli</i>	
				S	R	S	R	S	R
1	Aztreonam	ATM	30	72.6	27.4	65.8	34.2	78.7	21.3
2	Gentamicin	GEN	159	38.9	61.1	42.6	57.4	28.8	71.2
3	Amikacin	AMI	30	68.7	31.3	76.5	23.5	91.4	8.6
4	Ciprofloxacin	CIP	5	62.4	37.6	56.4	43.6	61.8	38.2
5	Ceftazidime	CAZ	30	58.7	41.3	78.7	21.3	77.6	22.4
6	Cefotaxime	CTX	30	43.1	56.9	77.6	22.4	85.9	14.1
7	Cefepime	FEP	10	55.9	34.1	83.7	16.3	81.9	18.1
8	Ampicillin	AMP	30	2.6	97.4	3.8	96.2	4.7	95.3
9	Piperacillin-tazobactam	TZP	20/10	73.9	26.1	55.3	44.7	56.2	43.8
10	Amoxicillin-clavulanate	AMC	20/10	12.2	87.8	19.9	80.1	32.1	67.9

S = Sensitive R = Resistant

In this study, *in vitro* time-kill assay was used to analysis combination of antibiotics with AgNPs against Gram-negative bacilli isolates. Two-drug combination of 1/2 MIC of ampicillin with 1/2 MIC of AgNPs displayed synergistic bactericidal effects against *P. aeruginosa* with markedly reduction in colony count after 24 h of incubation. Furthermore, two-drug combination of 1/2 MIC of amikacin with 1/2 MIC of AgNPs display synergistic activity against multidrug-resistant *P. aeruginosa* (Figure 5. A, B). In additions, the two-drug combinations of sub-MIC(1/2 MIC) of AgNPs with either 1/2 MIC of ampicillin or 1/2 of amikacin exhibited synergistic effect against *K. pneumoniae* and *E. coli* with clear inhibition of microbial growth curve after 6 h and 24h of incubation (Figure 5. C- E).



alone and in combination with antibiotics

The development of biofilms by Gram-negative bacteria lead the emergence of drug-resistant strains with challenge in treatment with traditional antibiotics. The capability of AgNPs to prevent the of biofilms formation were evaluated alone or in combination with ampicillin and amikacin against multidrug-resistant Gram-negative bacilli isolates. The results showed that AgNPs alone with $\frac{1}{2}$ MIC can inhibit biofilm formation about 20 - 25 % of Gram-negative bacilli, while combination of AgNPs with ampicillin exhibited notable inhibition of biofilms formation by 60 - 75 % of Gram-negative bacilli. Moreover, the combination of $\frac{1}{2}$ MIC of AgNPs with $\frac{1}{2}$ MIC of amikacin showed strong synergistic anti-biofilm activities and inhibition of biofilm formation approximately 75% to 80% of Gram-negative bacilli as indicated in figure 6.

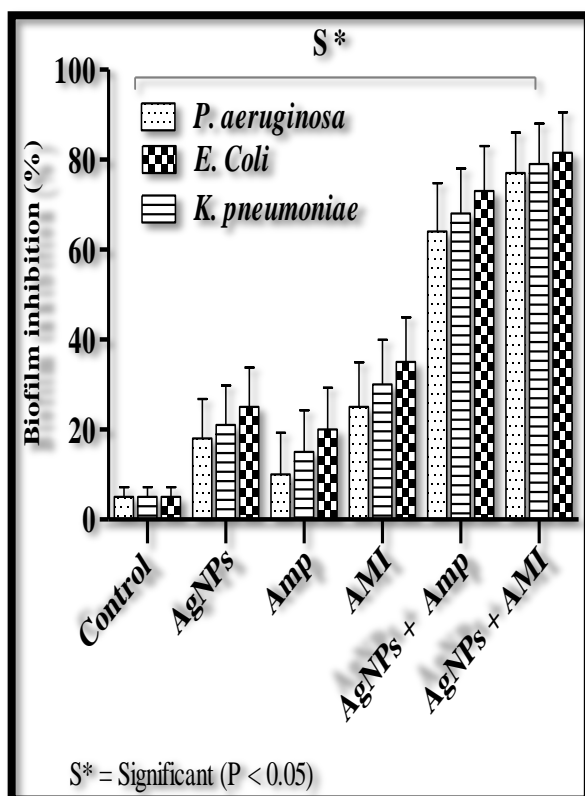


Figure 6. ant-biofilm inhibitory activity of AgNPs alone and in combination with antibiotics.

Discussion: burn wound infections mediated by multidrug resistant Gram-negative bacteria with bacterial biofilm development are often difficult to respond conventional antibiotics(12). Gram-negative bacilli are one of the most common bacteria isolated from burn wound infections. Numerous recent studies by Rajani M et al(13), Campbell W Ret al(14) , and Shankar Pet al(15)indicate that *Pseudomonas aeruginosa* was predominated Gram-negative bacilli isolated from burn wound infections followed by *Klebsiella pneumonia* or *Escherichia coli*.The results of the present study are confirmed that Gram-negative bacilli particularly *Pseudomonas aeruginosa* as the main source of burn wound infections. These bacterial pathogens are creating from the urogenital and gastrointestinal tracts or from upper respiratory system add to the hospital condition and environmental contamination. In this study, the average of bacterial resistance was very

high against selected antibiotics, the approach results are documented by Rashid K et al (16) in our country. High resistance rate of antibiotics by Gram-negative bacilli isolates may be due to widespread drug-resistant bacteria or self-medication. Gurunathan S et al (7) studied the antibacterial activity of silver nanoparticles in combination with different antibiotics "tetracycline, erythromycin, chloramphenicol, gentamicin, and vancomycin" against Gram-positive and Gram-negative bacterial pathogens, and they found strong synergistic bactericidal effect of AgNPs when combined with conventional antibiotics against :

Gram positive and Gram-negative bacteria "*Streptococcus pneumoniae* *Staphylococcus aureus*, *Shigella flexneri* and *Pseudomonas aeruginosa*" with inhibition of bacterial biofilm activity about 65%. A recent study by Barapatre A et al(17) , they found synergistic antimicrobial effect of AgNPs in combination with streptomycin and kanamycin, oxytetracycline against *Staphylococcus aureus*, *Escherichia coli* *Pseudomonas aeruginosa* with 80-90 % inhibition of bacterial biofilm formation. In this study, AgNPs showed remarkable antibacterial activity against multidrug-resistant Gram-negative bacilli isolated from burn wound infections, these results are consistent with numerous of studies have documented antibacterial activities of AgNPs against Gram-positive and Gram-negative bacterial pathogens with close results of MIC values (18, 19). In our experiments, combination of AgNPs with β -lactam antibiotic (Ampicillin) and aminoglycoside (Amikacin) antibiotics showed synergistic bactericidal effect and complete eradication of drug resistance Gram-negative bacilli with inhibition of biofilms formation by 60 - 75%. The synergistic antibacterial and anti-biofilm activity between AgNPs and selected antibiotics might be attributed to different mechanisms of action of antibiotics and AgNPs effect molecular targets different from selected drugs which including " i) Silver ions penetrate bacterial cell, denature ribosomes and suppress the expression of enzymes and proteins essential for ATP production, thus leading to cell disruption(20). ii) Silver has also the ability to prevent DNA unwinding by binding to them, hence inhibiting the replication of bacteria(21). iii) Targeting the bacterial membrane also leads to dissipation of proton motive force(22)". On the other hand, ampicillin is a beta-lactam antimicrobial drugs that assaults Gram-negative bacteria. The amino group in this antibiotic it has the ability to infiltrate external membrane of Gram- negative bacteria and suppression of transpeptidase formation, which is necessary for bacterial cell wall forming, and eventually leads to cell wall lysis. amikacin is semi-synthetic aminoglycoside antibiotic act as inhibitor for protein synthesis of bacteria. This suggests mechanism of interaction between and antibiotics might expand the applicability of AgNPs to control burn wound infections.

Conclusion: This study confirmed a synergistic bactericidal effects and enhanced anti-biofilm activity of AgNPs alone or in combination with broad-spectrum aminoglycoside (Amikacin) and β -lactam (Ampicillin) antibiotics against multidrug resistant Gram-negative bacilli (*Pseudomonas aeruginosa*, *Escherichia coli*, *klebsiella pneumoniae*) isolated from burn wound infections. The results propose that AgNPs could be applied as adjuvant in therapy of burn wound infections. Future studies are required on the molecular mechanisms of AgNPs and *in vivo* experiments to

overcome multidrug resistant bacteria causing burn wound infections.

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