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Effect of silver nanoparticles on the antibacterial activity of Levofloxacin against methicillin-resistant *Staphylococcus aureus*

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Abstract. – OBJECTIVE: The paper presents the antibacterial activity of silver nanoparticles (AgNPs) when conjugated with Levofloxacin. The AgNPs used in this study were synthesized from silver nitrate using sodium borohydride as a reducing agent.

MATERIALS AND METHODS: Levofloxacin activity was determined by minimum inhibitory concentrations (MICs) and also the erythrocyte hemolytic assay determined the capability of conjugation to cause hemolysis in human erythrocyte.

RESULTS: The synthesis of levofloxacin–AgNP conjugates was confirmed by ultraviolet/visible (UV/vis) spectroscopy. A peak absorption value between 400-450 nm for the extract and the color change to dark brown were corresponding to the plasmon absorbance of AgNPs. On the other hand, Levofloxacin–AgNPs could be effective against methicillin-resistant *Staphylococcus aureus* (MRSA). The MICs of levofloxacin and Levofloxacin–AgNPs were 12 and 10 μM, respectively.

CONCLUSIONS: These findings indicated that Levofloxacin-AgNPs had an effective bactericidal activity against the bacterial MRSAs. This conjugation appeared to inhibit bacterial adaptive capabilities, which leads to inhibition of bacterial resistance.

Key Words:
Levofloxacin, Silver nanoparticles, Minimum inhibitory concentration, Erythrocyte hemolytic.

Introduction

Recent treatment of human infections has facilitated the development of bacteria resistant to the use of particular antibiotics. Thus, a global increase in antimicrobial resistance is threatening the worldwide population[1]. The increase in resistant strains has been accompanied by a decrease in the number of antibiotics developed for clinical purposes. Over the past 30 years, as the number of antibiotics approved by the U.S. Food and Drug Administration to treat infections has declined by 90%, a number of global measures have been taken to prevent people from reach levels after using antibiotics[2]. For example, methicillin-resistant *Staphylococcus aureus* (MRSA). According to a recent World Health Organization (WHO) report about the development of bacterial resistance, the most type of bacteria causing the major types of resistant nosocomial infections is *S. aureus*. *S. aureus* is the strain that progresses to MRSA by interspecies transfer of the mecA gene from an ancestral *Staphylococcus* species to *S. aureus* mediated by a special staphylococcal mobile genetic element[3]. MRSA is a Gram-positive bacterium that causes a wide range of very dangerous human diseases. Nosocomial infections caused by MRSA strains have become a serious problem internationally as MRSA causes invasive infection, leading to an increased mortality rate of up to 20%. It causes inflammation in many tissues, septicemia, and even life-threatening infections. The risk of MRSA infections comes not only from the emergence of multi-drug resistant bacteria but also from the capability of bacteria to develop strong biofilm structures[4].

Nanotechnology has recently emerged as a rapidly growing field with many applications in biomedical sciences. At the same time, silver has been used as an antibacterial and antiseptic material with relatively no undesirable effects. Silver nanoparticles (AgNPs) have broad-spectrum antibacterial, antifungal, and antiviral properties. AgNPs can penetrate bacterial cell walls and cause structural changes in cell membranes and even result in cell death[5-7].

Levofloxacin belongs to the fluoroquinolone class of antibiotics that exhibits a bactericidal effect against both Gram-positive and -negative

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strains. Antimicrobial activity is mediated by inhibition of two types of topoisomerase enzymes, DNA gyrase, and topoisomerase IV[8,9]. In this study, we combined AgNPs with levofloxacin against the standard *S. aureus* and MRSA strains.

Materials and Methods

Bacterial Cultures

The bacterial strains used were acquired from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) and these include *S. aureus* (ATCC 29213), which was used as a control strain, methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-41), which was clinically isolated from a hospital, New York City, and 1994 MRSA was also employed in the study.

Synthesis of AgNPs Coated with Drugs

Levofloxacin-conjugated AgNps (Levo-AgNPs) were synthesized. Briefly, 5 mL (0.1 mM) levofloxacin solution was reacted with 5 mL (0.1 mM) silver nitrate solution, and the reaction mixture was magnetically stirred for 10 min. Twenty µL of 5 mM freshly prepared sodium borohydride solution ($NaBH_4$) was added to the reaction mixture. The color of the solution turned yellow-brown from clear upon addition of a reducing agent, which indicated the silver ion reduction and formation of Levo-AgNPs. NPs were centrifuged at 12,000 × g for 1 h after which the supernatant was collected, freeze-dried, and the unloaded drug concentration was determined by weighing. The results were expressed as percentage of the drug amount contained in 100 mg of the dried nanoparticle[10].

Characterization of AgNPs-Coated Drugs

After successful synthesis of nanoconjugates, the Levo-AgNPs were subject to complete analysis via ultraviolet/visible spectrophotometry (UV/vis)[6,11]. Absorption had shown of silver nanoparticles surface plasmon resonance at 420 nm (Figure 1).

Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBCs) Determination of Levofloxacin Alone

Minimum inhibitory and minimum bactericidal concentrations (MICs and MBCs, respectively) were determined against standard bacterial strains of *S. areues* and MRSA after testing each strain in different concentrations of each antibiotic (concentration ranged from 0.25 to 250 µM). Levofloxacin solution was prepared by dissolving it in water then diluted in the sterile broth[12].

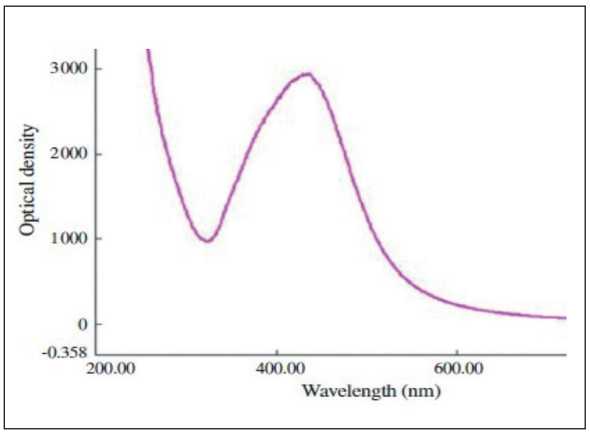

Figure 1. Absorption had shown of silver nanoparticles surface plasmon resonance at 420 nm.

Minimum Inhibitory Concentrations (MICs), and Minimum Bactericidal Concentrations (MBCs) Determination of the Active Peptides

Using sterile 96-well polypropylene microtiter plates, the microbroth dilution method outlined by the Clinical and Laboratory Standards Institute (CLSI) guidelines was adopted to determine MICs and MBCs of the active peptides. In brief, the Mueller Hinton Broth (MHB) was used as the growth medium for organisms after removing it from the stock media of frozen glycerol. Bacterial cells were grown overnight in MHB and diluted to 106 colony forming units (CFU)/ml in the same medium before use. Different dilutions with concentrations of Levo–AgNPs in the range of 0.5 to 100 µM as final concentrations were prepared. In 96-well microtiter plates, 50 µl of each combination concentration and 50 µl of diluted bacterial suspension were then added to each well. Each plate included six replicates of each peptide concentration divided over six wells. The plate was incubated for 18 h at 37ºC. Bacterial growth was then determined by measuring OD at $\lambda = 570$ nm using an enzyme-linked immunosorbent assay (ELISA) plate reader, and MIC was determined accordingly as the lowest concentration of antimicrobial drug needed to inhibit bacterial growth. Each plate included a positive control (50 µl of bacterial suspension plus 50 µl MHB without any antimicrobial agents) and a negative control (100 µl

Table I. The MIC value of Levofloxacin and Levo–AgNPs against both S. aureus (ATCC 29213), and MRSA (ATCC BAA-41).

Antibiotics	Levofloxacin–AgNPs	Levofloxacin
Staphylococcus aureus (ATCC 29213)	0.5 μM	2 μM
MRSA (ATCC BAA-41)	10 μM	12 μM

of MHB in each well) to ensure bacterial activity and sterility of MHB, respectively. Each experiment was repeated three times[12].

MBCs were determined by taking 10 μL from clear negative wells and from turbid positive control wells after which the aliquots were streaked on sterile labeled nutrient media[12].

Erythrocyte Hemolytic Assay

To investigate whether the conjugation process could cause hemolysis in human erythrocytes, hemolytic assays were performed. Two ml of human blood was placed into a 50-ml centrifuge tube, centrifuged at 3000 x g for 5 min. The supernatant was discarded, and the cell pellet was suspended in 48 ml of phosphate-buffered saline (PBS) and centrifuged at 3000 x g for 5 min; this step was repeated three times. The procedure was described by Maturana et al[13].

Statistical Analysis

Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) for Windows 16 statistical package program was used for statistical analysis of the data.

Results

In the beginning, the MRSA (ATCC BAA-41) strains were resistant toward the antibiotics, including levofloxacin. The MIC value of levofloxacin was 2 μM and Levo–AgNPs was 0.5 μM toward *S. aureus* (ATCC 29213). Levo–AgNPs appeared to have high antimicrobial activity against *S. aureus* (ATCC 29213) compared to free levofloxacin. MIC values decreased by approximately 25% against *S. aureus* (ATCC 29213). Also, the MIC value of levofloxacin was 12 μM and Levo–AgNPs was 10 μM against methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41). Levo–AgNPs appeared to have high antimicrobial activity against MRSA strains compared to free levofloxacin alone. MIC value decreased in the range of 16.7% against MRSA strains as shown in Table I. Moreover, the Levo–AgNPs antibacterial efficiency was higher than free levofloxacin against *S. aureus* (ATCC 29213) and MRSA strains. Levo–AgNPs did not cause hemolysis in the human erythrocytes *in vitro* as shown in Table II.

Discussion

‘‘Fight the resistance”, has become one of the most prevalent slogans used by all of the human healthcare communities worldwide because of the striking prevalence of emerging infectious diseases in addition to bacterial multi-resistance toward antibiotics that is escalating as reported recently. To address this mass issue, which has the potential to threaten humans, the development of new antimicrobial agents with a new mode of action can meet this imminent challenge[14].

Nanotechnology holds great promise in the biomedical field, particularly for diagnostics and drug delivery. Nanotechnology offers the possibility of delivering therapeutic agents to specific cells and receptors. Nanomaterial-based drug delivery systems have the potential to improve the pharmacokinetics and pharmacodynamics of drugs[15]. The smaller size of the NPs provides a larger surface area for maximum drug loading and better accessibility to specific targets. Recently, various drug-binding NPs have been developed to eradicate drug-resistant bacterial infections[16]. The most common metal carriers in NPs-based drug delivery systems contain iron oxide, gold, and silver for inertia and biocompatibility.

Table II. The hemolysis activity of Levo–AgNPs in human erythrocytes *in vitro*.

Hemolysis %	Concentration (μM)
0	5
0	10
0	20
0	40
0	60
0	80
1	100

In this study, the combination of levofloxacin and AgNPs shows a synergistic effect with an MIC value of 0.5 μM against controlled strains and 10 μM against MRSA. This result can be explained by AgNPs effects on the cell wall, which involves destroying the wall so that the entry of levofloxacin and path to its target in the nucleus is very easy[17]. Also, the toxicity study showed negligible toxicity against human cells over the entire concentration range from 0.5 to 100 μM. This study demonstrated that the Levo–AgNPs showed noticeable antibacterial potential and was also capable of producing extracellular AgNPs. Furthermore, it was shown that the drug–AgNPs complex has a profound synergetic antibacterial efficacy against *S. aureus* and *Escherichia coli* test strains in which this synergetic effect was augmented.

Conclusions

These findings indicated that levofloxacin-AgNPs had an effective bactericidal activity against the bacterial MRSAs. This conjugation appeared to inhibit bacterial adaptive capabilities, which leads to inhibition of bacterial resistance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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