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*Research Article*

# Biomedical Potentialities of Silver Nanoparticles for Clinical Multiple Drug-Resistant *Acinetobacter baumannii*

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Multidrug-resistant *A. baumannii* is increasingly recognized as a significant problem in hospitals and causes high morbidity and mortality. Here, we studied the antibacterial effects of AgNPs on clinically isolated multiple drug-resistant *A. baumannii,* and search for the potential antibacterial mechanism. Based on the results from the colony-forming unit (CFU) method, flow cytometry (FC), and a BrdU ELISA, we conclude that AgNPs can simultaneously induce apoptosis and inhibit new DNA synthesis in bacteria in a concentration-dependent manner. This study presents the first discussion of an antibacterial effect by AgNPs in clinically isolated, multidrug-resistant *A. Baumannii* and provides a new strategy for the use of silver nanoparticles in the multidrug-resistant *A. Baumannii* clinical problem.

## 1. Introduction

*Acinetobacter baumannii* is an aerobic, gram-negative coccobacillus that causes nosocomial human infections, particularly in immune-compromised individuals. Septicemia, meningitis, endocarditis, pneumonia, wound infection, and urinary tract infections can be caused by *A. baumannii* [1]. The strains were called multiple drug-resistant *Acinetobacter baumannii* (MDRAB), because they were resistant to at least three of five categories of common antibiotics. The five antimicrobial drugs include cephalosporins, green carbon mildew alkenes, $\beta$-lactamase inhibitor compound preparations, aminoglycosides, and quinolones. MDRAB infections have increased rapidly and have become a significant problem in hospitals, particularly in the intensive care unit [2]. More than 10% of hospital-acquired infections are caused by *A. baumannii* in the United States, leading to a mortality rate greater than 50% in patients with sepsis or pneumonia [3]. Serious health care-associated infections (HAIs) [4, 5] and serious infections in solid organ transplant (SOT) recipients [6] can also be caused by *A. baumannii*. MDRAB has now been reported worldwide, and outbreaks of infections with these bacteria have become a serious threat to global public health [7]. There is an urgent need to find effective treatments for MDRAB [8].

Silver is used as a strong inhibitor with a broad spectrum of antimicrobial activities against bacteria, fungi, and viruses. Nanometer-sized silver particles (AgNPs) have long been known to have an antibacterial effect. AgNPs are generally smaller than 100 nm, containing 20–15,000 silver atoms, and exhibit unusual physical, chemical, and biological properties [9–11]. AgNPs could be good candidates to treat MDRAB. We have illustrated that decreasing proliferation and increasing apoptosis are very important mechanisms of the AgNP antibacterial effect in standard strains of *E. coli* [12]. In this study, the same antibacterial effect was also found for the first time in clinically isolated MDRAB, and the mechanism for this antibacterial effect was also related to cell proliferation and apoptosis.

Table 1: Clinical characteristics of three blood isolated samples.

| Drug | #1 | | | #2 | | | #3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | KB* | MIC** | Sensitive/resistant | KB* | MIC** | Sensitive/resistant | KB* | MIC** | Sensitive/resistant |
| Piperacillin | | ≥128 | R | | ≥128 | R | | ≥128 | R |
| Piperacillin/tazobactam | | ≥128 | R | | ≥128 | R | | ≥128 | R |
| Ampicillin/sulbactam | | ≥32 | R | | 8 | S | | ≥32 | R |
| Imipenem | | ≥16 | R | | ≥16 | R | | ≥16 | R |
| Meropenem | | ≥16 | R | | ≥16 | R | | ≥16 | R |
| Ceftriaxone | | ≥64 | R | | ≥64 | R | | ≥64 | R |
| Ceftazidime/cefepime | | ≥64 | R | | ≥64 | R | | ≥64 | R |
| Gentamicin | | ≥16 | R | | ≥16 | R | | ≥16 | R |
| Tobramycin | | ≥16 | R | | ≥16 | R | | ≥16 | R |
| Amikacin | 11 | | R | 12 | | R | 11 | | R |
| Ciprofloxacin | | ≥4 | R | | ≥4 | R | | ≥4 | R |
| Levofloxacin | | ≥8 | R | | ≥8 | R | | ≥8 | R |
| Trimethoprim/sulfamethoxazole | | ≥20 | R | | ≥20 | R | | ≥320 | R |
| Minocycline | 18 | | S | 21 | | S | 17 | | S |

*mm; **$\mu$g/ml.

Table 2: Clinical characteristics of 38 patients with MDR-AB.

| Group | Total number (%) | $p$ value |
|---|---|---|
| *Gender* | | |
| Male | 21 (55.26%) | 0.359 |
| Female | 17 (44.47%) | |
| *Age* | | |
| 21-50 | 2 (5.26%) | |
| 51-60 | 5 (13.16%) | 0.001 |
| 60 more | 31 (81.58%) | |
| *Source* | | |
| Blood | 4 (10.53%) | |
| Sputum | 20 (52.63%) | |
| Swab | 3 (7.89%) | 0.001 |
| Drainage fluid | 4 (10.53%) | |
| Urea | 2 (5.26%) | |
| Others | 5 (13.16%) | |
| *Department* | | |
| Intensive care unit | 13 (34.21%) | |
| Hepatobiliary surgery | 5 (13.16%) | |
| Respirations | 6 (16.79%) | 0.016 |
| Ontological surgery | 4 (10.53%) | |
| Encephalopathy | 8 (22.22%) | |
| Others | 2 (2.56%) | |

Table 3: Drug-sensitive results for 38 patients with MDR-AB.

| Drug | Number ($n$) | Resistance rate (%) |
|---|---|---|
| Piperacillin | 38 | 100 |
| Piperacillin/tazobactam | 38 | 100 |
| Ampicillin/sulbactam | 34 | 89.5 |
| Imipenem | 38 | 100 |
| Meropenem | 38 | 100 |
| Ceftriaxone | 38 | 100 |
| Ceftazidime | 38 | 100 |
| Cefepime | 38 | 100 |
| Gentamicin | 35 | 92.1 |
| Tobramycin | 35 | 92.1 |
| Amikacin | 35 | 92.1 |
| Ciprofloxacin | 38 | 100 |
| Levofloxacin | 22 | 57.9 |
| Trimethoprim/sulfamethoxazole | 18 | 47.4 |
| Minocycline | 0 | 0 |

## 2. Materials and Methods

*2.1. Regents and Antibodies.* The AgNP solution with a diameter of 5~10 nm and concentration of 1000 ppm was from Shanghai Huzheng Nanotechnology Co. Ltd. The propidium iodide (PI) reagent (50 $\mu$g/ml) was from BD Co. The FITC-conjugated annexin V and PI kit were purchased from Dojindo Molecular Technologies Inc. The cell proliferation kit was purchased from Roche Co. All other chemicals were supplied by Aldrich and were used as received. The *E. coli* (ATCC25922), *Enterobacter cloacae* (ATCC700323), and *Pseudomonas aeruginosa* (ATCC27853) strains were purchased from the American Type Culture Collection (ATCC) and maintained in our laboratory. The clinical strains of *A. baumannii* were isolated from the phlegm, urine, blood, bile, or throat swabs of patients in the Nankai Hospital of Tianjin. The FACS buffer was prepared with 0.5% BSA, 2 mM EDTA, and 500 ml PBS. Luria-Bertani (LB) liquid medium and solid medium were prepared in our laboratory.

*2.2. Drug Sensitivity Measurement.* A total of 38 MDRAB isolates were collected from the Laboratory Science Department of Nankai Hospital. The ethics committee of the Tianjin

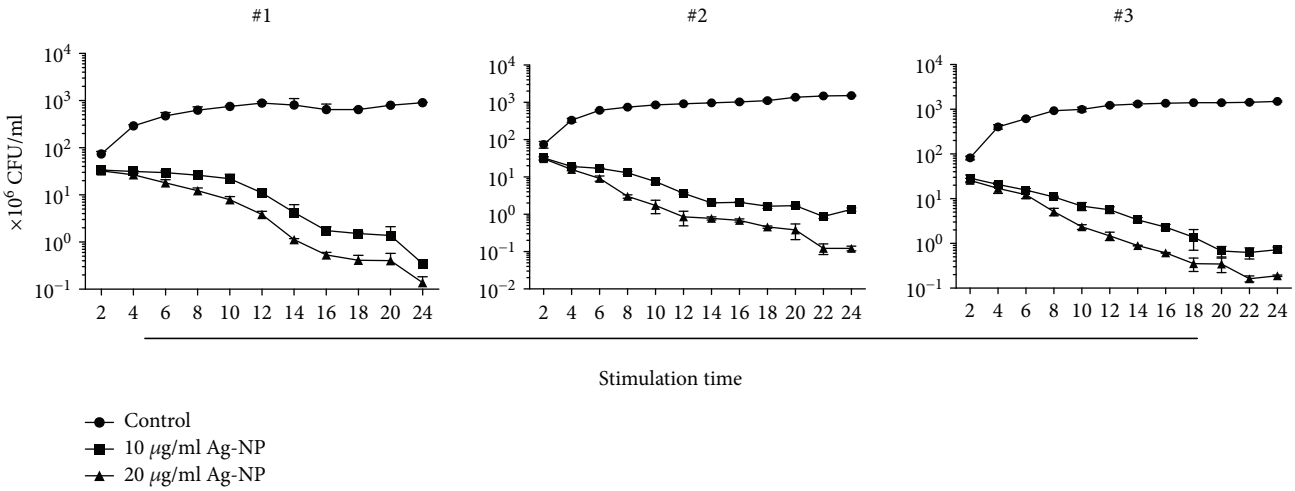


Figure 1: The toxicity of AgNPs to three selected MDR-AB as shown by CFU counting.

Medical University approved the use of human samples for this study. Clinical stains were tested by a VITEK 2 compact analytic system. *Enterobacter cloacae* was used as a control strain for identification purposes, and *Escherichia coli* and *Pseudomonas aeruginosa* were used as susceptibility control strains. The results were determined by the Clinical and Laboratory Standards Institute (CLSI) (2012) recommendations. The MIC was determined only for some of the antibiotics by the agar dilution method following CLSI recommendations (Table 1).

*2.3. Antibacterial Effect Testing.* The clinical samples were incubated in 5 ml of LB media at 37°C for 24 h. After cultivation, the cells were diluted (1 : 100) and incubated with 10 or 20 $\mu$g/ml AgNPs overnight. The bacterial concentrations were determined by counting colony-forming units (CFU). Each experiment was performed twice.

*2.4. Apoptosis Evaluation.* The clinical bacteria were cultured in 5 ml of LB medium with different concentrations of AgNPs (0, 5, 10, 15, 20, or 25 $\mu$g/ml) for 2 h. The apoptosis testing kit was performed according to the manufacturer's instructions. Data were collected by a FACSVerse instrument (BD). Experiments were performed in triplicate, and the data were analyzed using FlowJo software (http://www.flowjo.com/).

*2.5. Proliferation Testing.* The proliferation of the clinical isolated bacteria was determined by BrdU assay. Briefly, bacteria were cocultured with different concentrations of AgNPs (0, 5, 10, 15, 20, 25, or 30 $\mu$g/ml) and BrdU (10 $\mu$M) for 2 h. After incubation, the dye was incubated with 200 $\mu$l of sample and incubated for 30 min. The absorbance values were measured at 370 nm using a microplate reader (Biotek, USA). The experiments were performed in triplicate, and the growth curves were plotted using Prism 5 software.

*2.6. Statistical Analysis.* One-way ANOVA and Tukey's multiple comparison tests were used to determine whether the AgNPs had a significant effect on the clinical isolates. Statistically significant differences were identified when $p < 0.05$.

## 3. Results

*3.1. Characterization of Clinical Isolates.* 38 MDRAB clinical isolates were obtained from different types of specimens including sputum ($n = 20$), swabs ($n = 3$), blood ($n = 4$), drainage fluid ($n = 4$), urea ($n = 2$), and others ($n = 5$). Epidemiological analysis of the 38 patients with MDRAB revealed that 31 were ≥60 years old, 5 were between 51 and 60 years old, and 2 were between 21 and 50 years old. Among 21 males (55.26%) and 17 females (44.74%), 13 isolates were collected from the ICU, and 6 isolates were collected from the respiratory department. Compared with the total bacterial number, there were no significant differences found between different genders ($p = 0.359$), while the significant differences were found in different age ranges ($p = 0.000$), different departments ($p = 0.000$), and different sources ($p = 0.016$) (Table 2). But based on all the results, it was indicated that the problem of the MDRAB existed in the whole hospital, and the solution for that was really urgent.

Based on the results from the VITEK 2 compact analytic system, all 38 clinical isolated stains were resistant to at least eight or more antibiotics (Table 3). According to the definition of the multiple drug resistance of the bacteria, all the 38 clinical isolated samples are MDR-AB. All the samples were from six different sources. But four of them were isolated from the patients' blood. With the bacterial infection, it could be cleared by the immune system. If the bacteria entered into the blood, bacteremia could occur, which is very dangerous to the patients. It was indicated that the MDR-AB isolated from the blood could have the higher toxicity ability. So three different MDR-AB samples isolated from the blood were selected for the further study about the AgNP antibacterial effect.

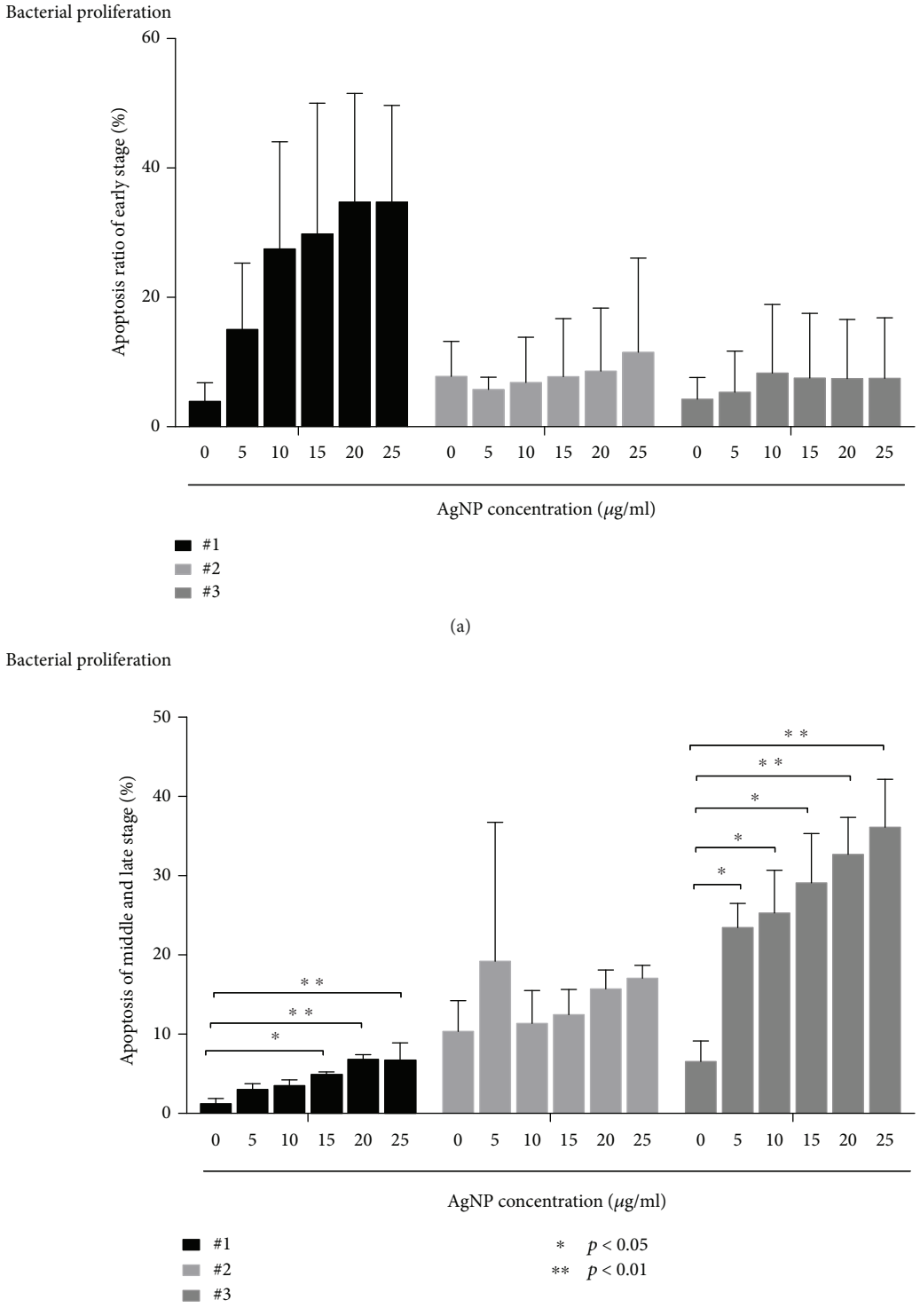


Figure 2: Flow cytometric analysis for bacterial apoptosis percentage using annexin V-PI staining. (a, b) Statistical analysis of bacterial apoptosis percentage by one-way ANOVA, followed Tukey's multiple comparison test ($n = 3$). (a) The percentage of apoptotic bacteria in the early stage, including the bacteria in the PI-negative, annexin V-positive quadrant. (b) The percentage of apoptotic bacteria in middle and late stages, including the bacteria in PI-positive, annexin V-positive quadrant.

*3.2. Antibacterial Resistance of Three Isolate Samples from Blood.* According to the results, the three selected blood-isolated samples are resistant to most of the antibiotics, while all were sensitive to minocycline (KB: #1 = 18 mm, #2 = 21 mm, and #3 = 17 mm) (Table 1). And besides that, #2 was also sensitive to ampicillin (MIC = 8 $\mu$g), which was from a 65-year-old male patient admitted to the ICU.

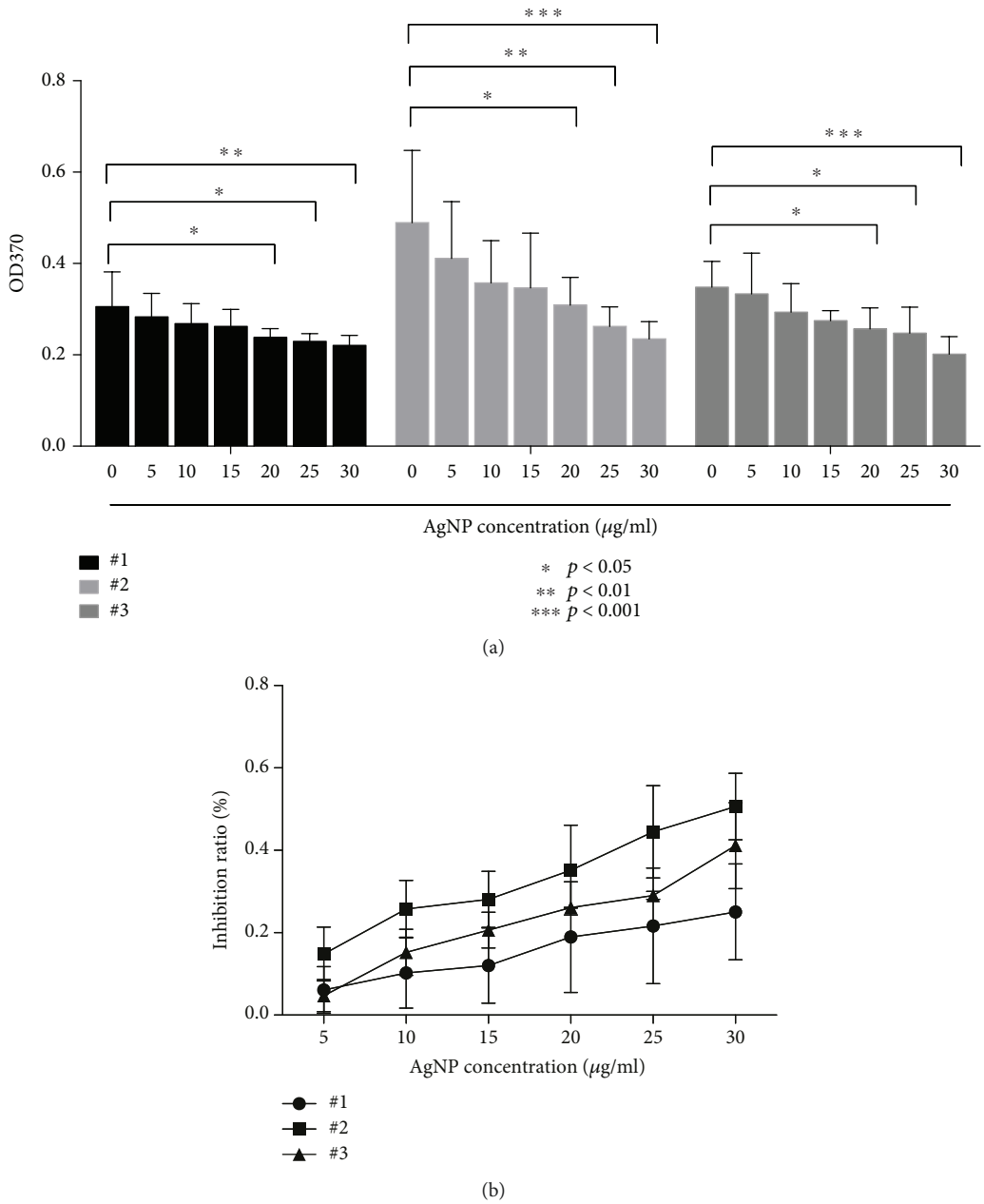


Figure 3: Analysis of new DNA synthesis by ELISA. (a) Growth inhibition results for different concentrations of AgNPs. (b) Growth inhibition rate for each isolate. All data were analyzed by one-way ANOVA and Tukey's multiple comparison test ($n = 3$).

*3.3. AgNP Inhibition Test for Three Selected Samples.* The inhibition testing was determined by measuring colony-forming units (CFU) every two hours after coincubation with different concentrations of AgNPs (10 or 20 $\mu$g/ml). Similar to our previous study in *E. coli* [12], the CFU results showed that all MDRAB clinical isolates were also sensitive to the AgNPs. The results of different incubation times with different AgNP concentrations demonstrated that compared with the control sample, the AgNPs were toxic to all three isolates (Figure 1).

Control samples, which were not treated with AgNPs, exhibited an increasing growth curve. At each time point, the AgNP-treated cells presented a lower CFU than the control sample. This decreasing trend for the treated sample was also positively correlated with the AgNP concentration for all three isolates from the blood, similar to *E. coli*. All of these results indicated that the AgNPs could decrease the number of MDRAB clinical isolates.

*3.4. Determination of Apoptosis in the Isolates.* The mechanism of the AgNP inhibition for clinical isolated blood sample was tested by analyzing apoptosis assay. Apoptosis can be divided into three different stages: early, middle, and late phases. According to the FC results, with the AgNP concentration increasing, both the early stage (Figure 2(a)) and the middle/late stages (Figure 2(b)) were increased. Statistical

analysis by one-way ANOVA followed by Turkey's test showed the statistical significance in middle/late apoptosis stage results. The $p$ values of the apoptosis rate between the control group and the stimulated groups were significant in isolates #1 and #3 for the middle and late stages of apoptosis (Figure 2(b)),

*3.5. Bacterial Proliferation.* A BrdU proliferation kit was used to monitor bacterial proliferation via the synthesis of new DNA. The results showed that the absorbance of the AgNP-treated bacteria (0, 5, 10, 15, 20, 25, or 30 $\mu$g/ml) was lower than that of the control group, and the reduction in new DNA synthesis was also related to the AgNP concentration (Figure 3(a)). Specifically, there was a particularly high sensitivity to AgNP shown in strain #2 (Figure 3(b)). These data were analyzed by one-way ANOVA, revealing a statistically significant difference between the stimulated and control groups.

## 4. Discussion

*Acinetobacter baumannii* is a major nosocomial pathogen with high morbidity and mortality in hospitalized patients. In recent years, with the development of antibody resistance, *A. baumannii* has become a serious clinical problem [13–15]. It has emerged as a leading cause of nosocomial infections, especially in intensive care units (ICUs), causing a variety of infections including septicemia, urinary tract infections, and wound infections. Infections and outbreaks caused by multidrug-resistant *A. baumannii* (MDRAB) are prevalent and have been reported worldwide over the past twenty years [16]. It is important to solve this MDRAB problem.

Many synthetic polymers, such as metals and metallic alloys, are studied for their biomedical applications [17–19]. Among the metals, AgNPs have been widely used as antimicrobial agents against bacteria, fungi, and viruses [20, 21]. AgNPs also had been confirmed as an effective antibacterial agent for various gram-negative and gram-positive bacteria [22]. The mechanisms for the antibacteria are involved inducing cell death and the ROS generation. But most of the studies were focused on the standard bacterial strain. In our paper, we used high toxicity clinical-isolated MDR-AB for the antibacterial study, and we believed the results should be more helpful for the further clinical application usage.

Clinical MDRAB strains were collected from different types of specimens from different hospital departments in our study (Table 2), which more accurately represents standard clinical strains commonly used in laboratories. All the isolates were multidrug-resistant (Table 3). Three isolates from the blood were chosen for the further detection of antibacterial effects (Table 1). In our work, the growth curves of MDRAB exposed to AgNPs, as shown in Figure 1, indicate that AgNPs inhibit the number of clinical MDRABs, and this inhibition trend is positively related with the concentration of AgNPs. All the results confirmed the AgNPs also had the same antibacterial activity on the clinical isolated strain.

This antibacterial effect is like our previous study in *E. coli* [12]. In our previous study, we demonstrated the toxicity of AgNPs to *E. coli* via induction of bacterial apoptosis and inhibition of DNA synthesis, and the extent of these effects was positively related to the AgNP concentration. Therefore, we determined if the same mechanisms were also involved in the toxicity of AgNPs to clinical MDRABs. According to the results from Figure 2, AgNPs induced apoptosis in the clinical MDRABs, and this activity increases with increasing AgNP concentration. The analysis of each stage of apoptosis showed the same conclusion: high concentration of AgNP can induce greater apoptosis. Bacterial proliferation was analyzed by the BrdU ELISA kit. According to the results, bacterial proliferation was inhibited by the AgNPs (Figure 3(a)). The tread of this inhibition rate was also strongly related to the nanoparticle concentration (Figure 3(b)).

In conclusion, we demonstrated that the toxicity of AgNPs to MDRABs is also related to bacterial apoptosis and proliferation. These conclusions can be used to develop new antibacterial strategies for the MDRAB problem in the clinic by using AgNPs.

## Data Availability

All the data used to support the findings of this study are included in the article.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

XY, MC, and QY conceived and designed the experiments as well as performed the experiments. HB and QY analyzed the data. XY, MC, QH, and XL contributed reagents/materials/analytical tools. HB and QY wrote the paper. HB and XC supervised the work. Minghui Chen and Xiaoxu Yu contributed equally to this work.

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