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Antibacterial Activity of Silver-nanoparticles Against *Staphylococcus aureus* and *Escherichia coli*

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The antibacterial activities of silver nanoparticles (Ag-NPs) were studied with respect to Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* by observing the bacterial cells treated or not with Ag-NPs by field emission scanning electron microscope (FE-SEM) as well as measuring the growth curves, formation of bactericidal reactive oxygen species (ROS), protein leakage, and lactate dehydrogenase activity involved in the respiratory chain. Bacterial cells were treated with Ag-NPs powder, and the growth rates were investigated under varying concentrations of Ag-NPs, incubation times, incubation temperatures, and pHs. As a result, *S. aureus* and *E. coli* were shown to be substantially inhibited by Ag-NPs, and the antibacterial activity of Ag-NPs did not fluctuate with temperature or pH. These results suggest that Ag-NPs could be used as an effective antibacterial material.

Key words: *Staphylococcus aureus*, *Escherichia coli*, silver nanoparticles (Ag-NPs), antibacterial activity

Introduction

Human beings are often infected by microorganisms such as bacteria, molds, yeasts, and viruses present in their living environments. Because of the emergence and increase in the number of multiple antibiotic-resistant microorganisms and the continuing emphasis on health-care costs, many scientists have researched methods to develop new effective antimicrobial agents that overcome the resistances of these microorganisms and are also cost-effective. Such problems and needs have led to resurgence in the use of silver-based antiseptics that may be linked to a broad-spectrum activity and considerably lower propensity to induce microbial resistance compared with those of antibiotics [12, 20, 25, 26]. In particular, silver ions have long been known to exert strong inhibitory and bactericidal effects as well as to possess a broad spectrum of antimicrobial activities [2]. Silver ions cause the release of K^+ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes and DNA, is an important target site of silver ions [8, 19, 22, 24].

When bacterial growth was inhibited, silver ions were deposited into the vacuole and cell walls as granules [4]. They inhibited cell division and damaged the cell envelope and cellular contents of the bacteria [23]. The sizes of the bacterial cells increased, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers exhibited structural abnormalities. In addition, silver ions can interact with nucleic acids [31]; they preferentially interact with the bases in the DNA rather than with the phosphate groups, although the importance of this mechanism in terms of their lethal action remains unclear [11, 21, 32].

The possibility of free-radical involvement in the antibacterial activity of silver nanoparticles (Ag-NPs) has been previously reported [14], but the underlying mechanism and characteristics remain unclear. The interaction between reactive oxygen species (ROS) and bacterial cell death was revealed in previous study [5]. According to the study, bacterial DNA or mitochondria can be affected by ROS. Thus, for instance, some of them show good antibacterial and antiviral effects, producing ROS such as superoxide anion (O_2^-), hydroxyl radical (OH·) and singlet oxygen

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($^1O_2$) with subsequent oxidative damage.

The effects of silver nanoparticles (Ag-NPs) on bacterial cell are complicate. However, direct morphological observation by electro-microscope gives us structural change on the bacterial cell. It may give us useful information for understanding antibacterial activity of silver nanoparticles.

Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli* were widely used to bacterial experiment. *S. aureus* and *E. coli* live on the body surface of mammals and sometimes occur infection to them. Furthermore, they show their unique cell envelope structure of Gram positive and Gram negative bacteria. Therefore, *S. aureus* and *E. coli* strains were selected for this antibacterial study.

In this study, silver nanoparticles (Ag-NPs) were evaluated for their applicability in increasing antibacterial activities against *S. aureus* and *E. coli*.

Materials and Methods

Materials, reagents, strains, and cultivation

The silver nanoparticles (Ag-NPs) powder used in this study was manufactured by Thermolon Korea, Inc. (Busan, Korea). The powder containing 5% silver was silver-brown and insoluble. Mueller-Hinton broth (Becton Dickinson, U.S.A.) and Mueller-Hinton agar (Becton Dickinson, U.S.A.) were used as culture media. Mueller-Hinton broth contains beef extract powder, an acid digest of casein, and soluble starch. Mueller-Hinton agar contains agar in addition to the above-mentioned ingredients. A protein determination kit and lactate dehydrogenase (LDH) cytotoxicity assay kit were purchased from Cayman Chemical Co. (Michigan, U.S.A.). Glutaraldehyde, osmium tetroxide, and 2',7'-dichloro-fluorescein were purchased from Sigma-Aldrich Co. (St. Louis, U.S.A.). All other reagents used were of the purest grade commercially available.

The *S. aureus* and *E. coli* cells used in the present study were supplied by Busan Paik hospital, Inje university (Busan, Korea).

Assaying the minimum inhibitory concentration of Ag-NPs

The minimum inhibitory concentration (MIC) of Ag-NPs was determined using the plate count method [18, 30]. The Ag-NPs powder was sterilized with UV radiation for 1 h, and the weight of the powder was then measured under aseptic conditions. Further, Mueller-Hinton broth containing 10^5 CFU/ml of bacterial cells was used as a culture medium. The final concentrations of Ag-NPs were 0, 50, 100, 150, 250, and 500 µg/ml. The medium was cultured in a shaking incubator at 37°C for 24 h, and the cultured media (100 µl) was spread onto Muller-Hinton agar and incubated at 37°C for 24 h. After incubation, the number of colonies grown on the agar was counted [30].

Determining the growth curves of bacterial cells exposed to different concentrations of Ag-NPs

To examine the growth curves of bacterial cells exposed to Ag-NPs, Mueller-Hinton broth with different concentrations of Ag-NPs powder (0, 50, 100, and 150 µg/ml) was used, and the bacterial cell concentration was adjusted to 10^5 CFU/ml. 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.

Determining the growth curves of bacterial cells exposed to Ag-NPs under different temperature and pH conditions

To determine the growth curve of bacterial cells exposed to Ag-NPs at various temperatures, different incubation temperature conditions were used [15]. The concentration of Ag-NPs in the broth was adjusted to 100 µg/ml, and the bacterial cell concentration was adjusted to 10^5 CFU/ml. For determine growth curves of bacterial cells under different temperature condition, each culture was incubated in a shaking incubator at 17, 25, or 37°C for 24 h. Culture broths with different pH conditions (pH 5.6, 7.2, and 8.2) were used to determine the growth curve of bacterial cells exposed to Ag-NPs at various pH conditions [15]. After incubation, the bacterial cell concentrations in the media were determined by measuring the O.D. at 600 nm.

Detection of reactive oxygen species (ROS)

The ROS formed by Ag-NPs was identified using 2',7'-dichlorofluorescein diacetate (DCFDA) [16]. The concentration of Ag-NPs treated was 100 µg/ml, and the number of bacterial cells used was adjusted to 10^5 CFU/ml. After all cultures were incubated at 37°C for 3 h, they were centrifuged at 4°C for 30 min at $300 \times g$, and then each supernatant was treated with 100 µM DCFDA for 1 h. The ROS formed in the sample was detected at 485/20 nm of fluorescence excitation wavelength, and 528/20 nm of

emission wavelength using Fluorescence Multi-Detection Reader (BIOTEK, U.S.A.).

Assaying the effect of Ag-NPs on protein leakage from bacterial cell membranes

Protein leakage from bacterial cells was detected using Bradford's protein assay [3]. The concentration of Ag-NPs was adjusted to 100 µg/ml, and the concentration of bacterial cells was 10^5 CFU/ml. Each culture was incubated in a shaking incubator at 37°C for 6 h. 1 ml of culture sample was obtained from each culture. The sample was centrifuged at 4°C for 30 min at 300 × g, and the supernatant was frozen at -20°C [10]. The supernatant was treated with Bradford's assay reagent, and the O.D. was measured at 595 nm.

Assaying the effect of Ag-NPs on respiratory chain LDH activity in bacterial cells

The LDH activity was determined by measuring the reduction of NAD^+ to NADH and H^+ during the oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses NADH and H^+ to catalyze the reduction of a tetrazolium salt to a highly colored formazan. The concentration of Ag-NPs was adjusted to 100 µg/ml, and that of bacterial cells was adjusted to 10^5 CFU/ml. Each culture was incubated in a shaking incubator at 37°C for 6 h. The culture was centrifuged at 4°C for 30 min at 300 × g, and the supernatant was discarded. The pellet was washed twice and then treated with LDH reaction solution in a microplate [11, 28]. The plate was incubated with gentle shaking on an orbital shaker for 30 min at room temperature. After incubation, the O.D. of the plate was determined at 490 nm.

Observing bacterial cells through field emission scanning electron microscope (FE-SEM)

To directly observe the morphological changes of bacterial cells treated or not with Ag-NPs, FE-SEM was used. Bacterial cells (10^6 CFU/ml) were treated with 100 ug/ml of Ag-NPs for 3 h, and centrifuged at 300 × g for 30 min. The pellets were washed with phosphate buffered saline (PBS) three times and pre-fixed with 2.5% glutaraldehyde for 30 min. The pre-fixed cells were washed with PBS two times and post-fixed with 1% osmium tetroxide for 1 h. After washing with PBS three times, dehydration process was conducted with 30, 50, 70, 80, 90 and 100% of ethanol. The fixed cell was dried and gold-coated using ion sputter (E-1030, Hitachi, Japan). The pre-treated samples were observed by FE-SEM (S-4300SE, Hitachi, Japan).

Statistical analysis

All experiments were repeated at least three times. The results were represented as means ± SD. All experimental data were compared using Student's t-test. A p-value less than 0.05 was considered statistically significant.

Results and Discussion

Minimum inhibitory concentration of Ag-NPs

To determine the lowest concentration that completely inhibited visible growth, the minimum inhibitory concentration (MIC) was used. The MIC of Ag-NPs against *S. aureus* and *E. coli* are shown in Fig. 1, showing that the MIC of Ag-NPs against *S. aureus* and *E. coli* was 100 µg/ml. When

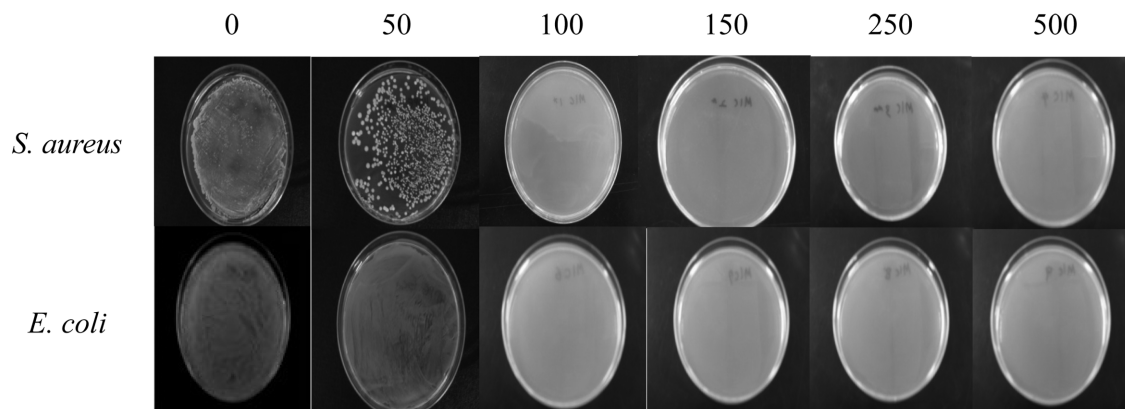| | 0 | 50 | 100 | 150 | 250 | 500 |

Fig. 1. Minimum inhibitory concentration (MIC, µg/ml) of Ag-NPs.

100 and 150 μg/ml Ag-NPs powder were used, growth was inhibited; however, when 50 μg/ml Ag-NPs was used, growth was only slightly inhibited. The antibacterial activities of the Ag-NPs against the Gram-positive *S. aureus* and Gram-negative *E. coli* were almost identical.

Growth curves of bacterial cells treated with different concentrations of Ag-NPs

The growth curves of bacterial cells treated with Ag-NPs indicated that Ag-NPs could inhibit the growth and reproduction of bacterial cells. The growth curves of Ag-NPs treated *S. aureus* cells are shown in Fig. 2(A). The bacterial growths of cells treated with 100 and 150 μg/ml Ag-NPs were inhibited. After 4 h, almost all treated bacterial cells were dead. The bacterial growth of the cells treated with 50 μg/ml Ag-NPs was also slightly lower than that of cells in the control group. The growth curve of *E. coli* cells treated with Ag-NPs is shown in Fig. 2(B). The bacterial growths of cells treated with 100 and 150 μg/ml Ag-NPs were inhibited. After 3 h, almost all bacterial cells in these groups were dead. As shown in Fig. 2, the growth of cells treated with 50 μg/ml Ag-NPs was also slightly lower than

that of cells in the control group. These findings indicate that the antibacterial activity of 50 μg/ml of Ag-NPs could slightly inhibit bacterial growth but not enough to outpace the speed of reproduction of the bacterial cells. Interestingly, upon comparison of the bacterial growth curves, the growth curves of the Ag-NP-treated bacteria indicated a faster growth inhibition of *E. coli* than of *S. aureus*.

Growth curves of bacterial cells exposed to Ag-NPs at different temperatures and pH

The growth curves of Ag-NPs treated *S. aureus* and *E. coli* cells at 17°C and 25°C did not differ from those of cells at 37°C (data not shown). The extent of growth inhibition in cells treated with Ag-NPs and grown at 17°C and 25°C as almost the same as in those grown at 37°C. However, the growth of cells in the control group was slightly lower than that of cells incubated at 37°C. The growth curves of Ag-NPs treated *S. aureus* and *E. coli* cells incubated at pH 5.6 and 8.2 did not differ from those of cells grown at pH 7.2 (data not shown). The rate of growth inhibition in cells treated with Ag-NPs and grown at pH 5.6 and 8.2 were almost the same as that in cells grown at pH 7.2. The growth rate of *S. aureus* cells in the control group held at pH 5.6 was slightly lower than that of cells incubated at pH 7.2.

Formation of ROS from bacterial cells treated with Ag-NPs

Recently, it was reported that the antibacterial activity of Ag-NPs is related to the formation of free radicals [14]. Furthermore, the free radicals generated by the Ag-NPs induce bacterial cell membrane damage. Some researchers have reported that ROS can exist naturally in intracellular and extracellular locations [6]. Under certain conditions, high levels of ROS can increase oxidative stress in cells. Oxidative stress can not only cause damage to the cell membrane, but can also cause damage to the proteins, DNA, and intracellular systems such as the respiratory system. In this study, ROS was measured using DCFDA (Fig. 3). After 3 h incubation, significantly increased ROS was detected in Ag-NPs treated group of *S. aureus* or *E. coli* but not in control group. These results indicate that Ag-NPs can form ROS with water, and so bacterial cell membrane, protein structure and intracellular system can be damaged owing to the ROS formed by Ag-NPs.
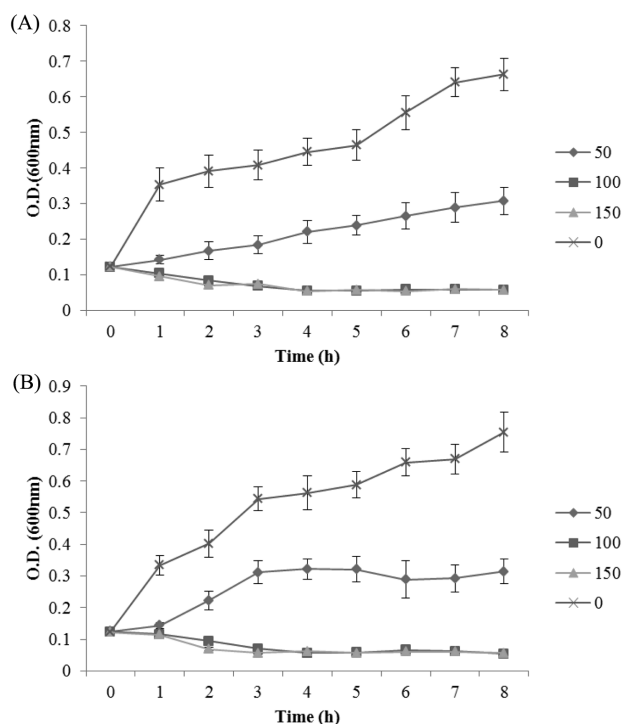

Fig. 2. Growth curves of *S. aureus* (A) and *E. coli* (B) cells exposed to different concentrations (μg/ml) of Ag-NPs at normal condition.
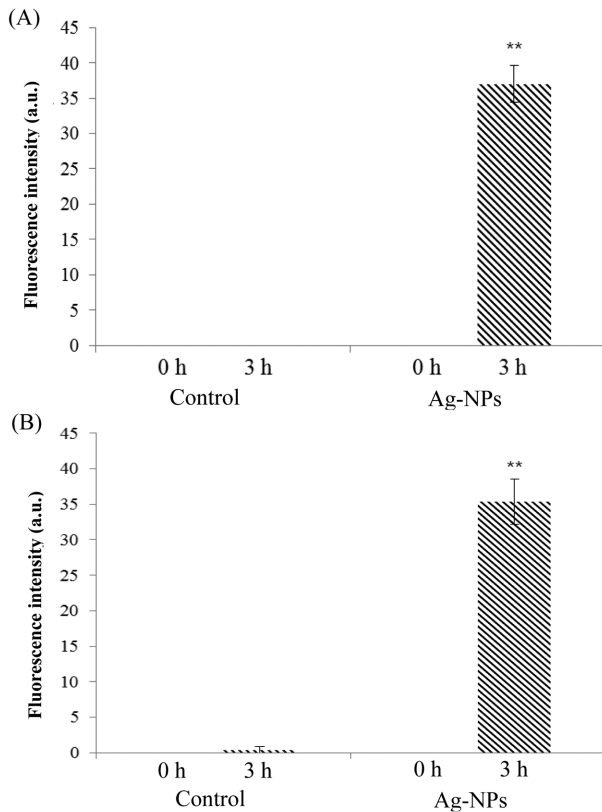
(A)

(B)

Fig. 3. Formation of ROS in *S. aureus* (A) or *E. coli* (B) cells exposed to Ag-NPs. The Ag-NPs group was treated with Ag-NPs at the concentration of 100 μg/ml for 0 h or 3 h, and the control was not treated.
**Significantly different from control group ($p < 0.01$).

(A)

(B)

Fig. 4. Leakage of protein from *S. aureus* (A) or *E. coli* (B) cells exposed to Ag-NPs. The Ag-NPs group was treated with Ag-NPs at the concentration of 100 μg/ml, and the control was not treated. *,**Significantly different from control group (*: $p < 0.05$; **: $p < 0.01$).

Effect of Ag-NPs on protein leakage from bacterial cell membranes

It was found that Ag-NPs could enhance protein leakage by increasing the membrane permeabilities of *S. aureus* and *E. coli* cells (Fig. 4). Initially, protein leakage from the membranes of *S. aureus* cells treated with Ag-NPs was almost the same as that from cells in the control group. At 6 h after incubation, protein leakage from cells treated with Ag-NPs considerably increased; however, there was no change in the amount of protein leakage from cells in the control group. Leakage from cells treated with Ag-NPs was significantly higher than that from cells in the control group. Furthermore, the initial protein leakage from the membranes of *E. coli* cells treated with Ag-NPs was almost the same as that from cells in the control group. At 6 h after incubation, protein leakage from *E. coli* cells treated with Ag-NPs was significantly increased compared to that from cells in the control group, indicating that Ag-NPs can
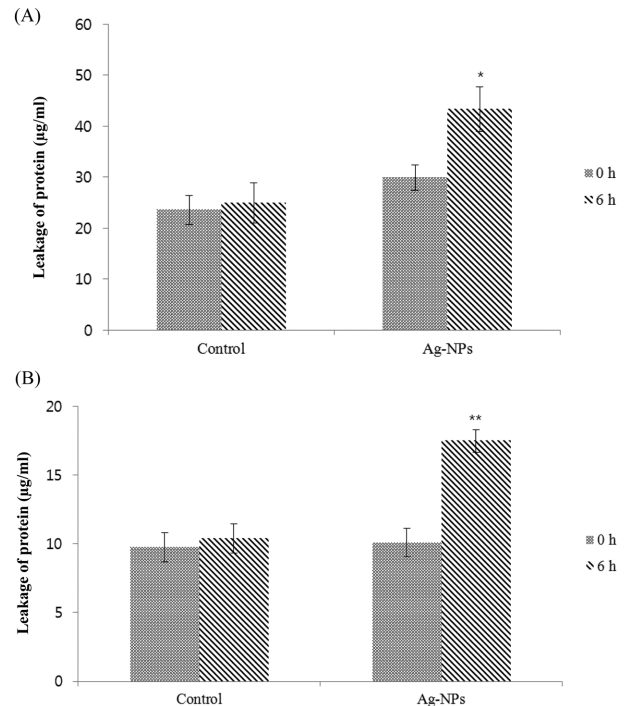
increase membrane permeability. Notably, higher amounts of proteins leaked through the *E. coli* membranes compared to those through the *S. aureus* membranes, suggesting that the antibacterial sensitivity of the Gram-positive *S. aureus* was lower than that of the Gram-negative *E. coli*. This difference was possibly attributable to the thickness of the peptidoglycan layer of *S. aureus*; an essential function of the peptidoglycan layer is to protect against antibacterial agents such as antibiotics, toxins, chemicals, and degradative enzymes. This result was consistent with the results of previous studies [7, 13].

Effect of Ag-NPs on respiratory chain lactate dehydrogenase activity in bacterial cells

To determine oxidative stress-induced damage of the respiratory system of the cells, LDH activity was measured. The effects of Ag-NPs on LDH activities of *S. aureus* and *E. coli* cells are shown in Fig. 5. The LDH activity of *S. aureus* cells in the control group increased with time, whereas the LDH activity decreased slightly in the cells treated with Ag-NPs. LDH activity in cells treated with Ag-
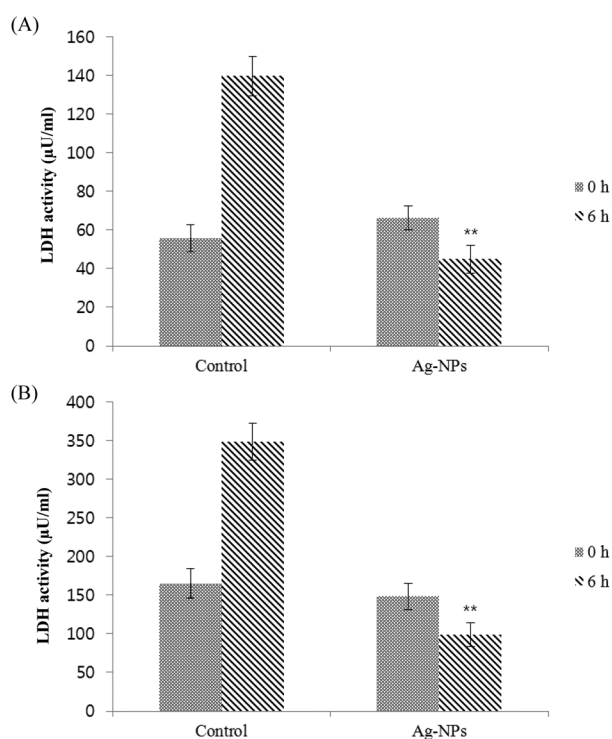
(A)

(B)

Fig. 5. Effect of Ag-NPs on respiratory chain lactate dehydrogenases in *S. aureus* (A) or *E. coli* (B). The Ag-NPs group was treated with Ag-NPs at the concentration of 100 μg/ml, and the control was not treated.
**Significantly different from Control group ($p < 0.01$).

NPs was significantly lower than that in cells of the control group. Moreover, the LDH activity of *E. coli* cells in the control group increased considerably with time, whereas that of the cells treated with Ag-NPs decreased slightly; there was a significant difference in the LDH activities in Ag-NPs treated cells and in cells of the control group. These results indicate that ROS formed by Ag-NPs inhibit LDH, an important enzyme in cellular respiration. As a result, Ag-NPs cause inhibition of bacterial growth and reproduction, in agreement with a previous study, which showed that the respiratory chain activity in *E. coli* was inhibited by Ag-NPs [17].

Morphological changes of bacterial cells treated with Ag-NPs

The morphological changes of bacterial cells were observed by FE-SEM (Fig. 6). In *S. aureus* cells, cells of control group were typically grape-shaped. The cell surface was intact and damage was not seen. However, in the cells of Ag-NPs treated group, there are many fragments on the

cell surface, indicating the damage of cell surfaces. In *E. coli* cells, cells of control group were typically rod-shaped. Each cell size was almost same and damage on the cell surface was not detected. However, in Ag-NPs treated group, instead of normal rod-shaped cells, irregular fragments appeared. Increased permeability of the cell membrane or leakage of cell contents could be caused by ROS [1, 6, 27]. Interestingly, morphological changes on the bacterial cells by Ag-NPs were different from those of previous studies [7, 15]. Whereas they showed only changes of cell surface due to increased permeability, this study showed not only morphological changes of cell surface but also cell fragments formed through damage of cell membranes. The cell fragments could be the products derived from leakage of cytoplasmic contents in damaged cells.

Some researchers have reported that the antimicrobial effect of the Ag-NPs on Gram-negative bacteria was dependent on the concentration of Ag in the nanoparticles and was closely related to the formation of "pits" in the cell walls [1, 27]. Further, negatively charged Ag-NPs accumulated in the bacterial membrane increased the permeability of the membrane.

The results of increased cell membrane permeability of this study can be evidence for the formation of Ag-NPs "pits" in the cell walls. Likewise, reduced LDH activity also can be results of formation of ROS species by Ag-NPs. However, interestingly, there are some difference between Gram positive *S. aureus* and Gram negative *E. coli*. *S. aureus* shows slightly less bactericidal activities in growth curves, protein leakage, and inactivation of LDH than *E. coli*'s. Especially, in case of FE-SEM observation, morphological destruction of bacterial cell of *S. aureus* was feeble than *E. coli*. This difference was possibly attributable to the difference of the peptidoglycan layer of the bacterial cell between Gram positive *S. aureus* and *E. coli*; an essential function of the peptidoglycan layer is to protect against antibacterial agents such as antibiotics, toxins, chemicals, and degradative enzymes [29]. The Gram negative cell envelope consists of outer membrane, thin peptidoglycan layer, and cell membrane. Beside to this, Gram positive cell envelope consists of lipoteichoic acid containing thick peptidoglycan layer and cell membrane. Whereas Gram negative peptidoglycan is only a few nanometers thick, Gram positive peptidoglycan is 30 through 100 nm thick and contains many layers. The thick peptidoglycan layer of Gram positive bacteria may protect formation of pits or
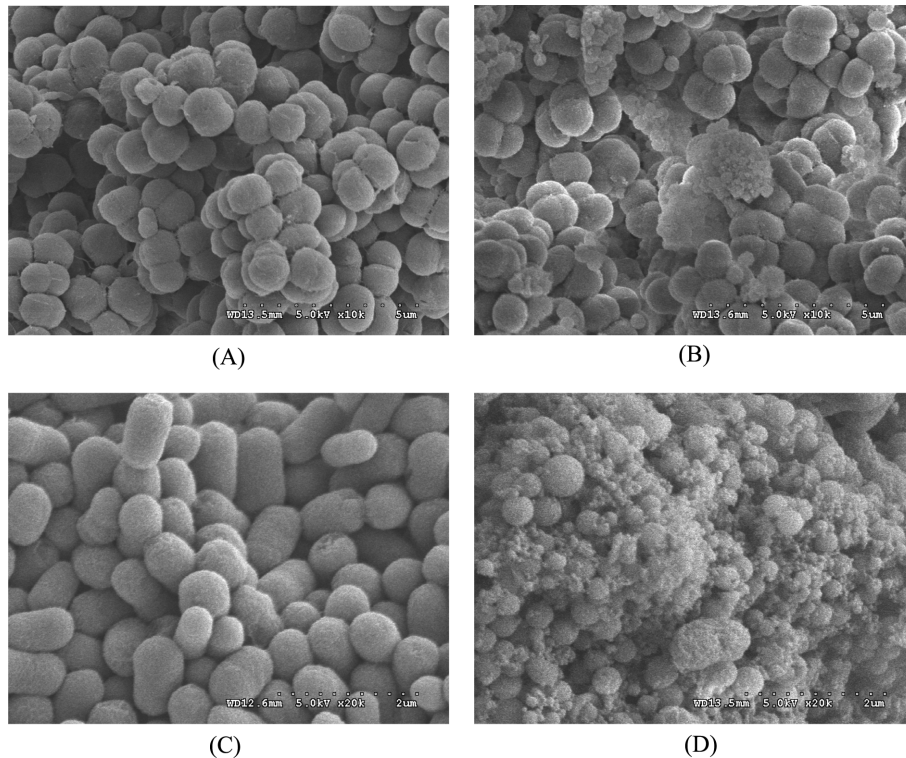
Fig. 6. FE-SEM micrograph of *S. aureus* **(A, B) and** *E. coli* **(C, D).** (A, C : control; B, D : treated with Ag-NPs).

ROS by Ag-NPs more severely than thin peptidoglycan layer of Gram negative bacteria.

In the present study, we demonstrated the antibacterial activities of Ag-NPs against *S. aureus* and *E. coli* by determining the growth curves of Ag-NPs treated bacterial cells, the stabilities of the antibacterial activity under various pH and temperature conditions, the protein leakage caused by increased membrane permeability, and the inactivation of LDH due to the formation of ROS induced by Ag-NPs.

In conclusion, this study showed that Ag-NPs have potent antibacterial activities against *S. aureus* and *E. coli* cells. The growth and reproduction of Ag-NPs treated bacterial cells were quickly inhibited. The various pH and temperature conditions did not affect the growth of Ag-NPs treated cells. Active formation of bactericidal ROS by Ag-NPs was detected. The inactivation of LDH and increased protein leakage observed with Ag-NPs treatment decreased the growth and reproduction of bacterial cells. Furthermore, the morphological changes on bacterial cells by Ag-NPs were observed by FE-SEM. This study indicates that Ag-NPs can be used as effective antibacterial materials against various microorganisms which can endanger human beings.

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국문초록

황색 포도상구균과 대장균에 대한 은나노 입자의 항균활성

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　본 연구는 은나노 입자의 항균활성을 알아보기 위하여, 그람 양성세균인 황색포도상구균과 그람 음성세균인 대장균에 대한 은나노 입자(Ag-NPs)를 처리 후, 세균세포 생장곡선측정, 활성산소생성능 측정, 세포질 단백질 누출량 측정, 젖산탈수소효소 활성측정 및 고분해능 임계방사 주사전자현미경 관찰이 수행되었다. 세균세포의 생장곡선 측정은 다양한 농도, 배양시간, 배양온도 및 pH에서 수행되었다. 결과적으로 황색 포도상구균과 대장균은 배양온도와 pH에 영향을 받지않고 은나노 입자에 의해 효과적으로 생장억제가 이루어지는 것을 관찰할 수 있었다. 또한 활성산소의 생성에 의하여 세포막의 파괴로 세포질내 물질의 유출을 세포질 유래 단백질 측정으로 확인할 수 있었으며, 젖산탈수소효소 활성측정을 통하여 은나노 입자에 대한 세포호흡억제활성 또한 확인할 수 있었다. 임계방사 주사전자현미경 관찰결과 은나노 입자에 의한 세균 세포표면의 형태학적 변화 또한 관찰되었다. 이러한 결과를 통하여 은나노 입자를 효과적인 항균활성소재로 활용 가능함이 입증되었다.