J Vet Clin 28(6) : 576-580 (2011)

Journal of Veterinary Clinics
www.ksvc.or.kr

Morphological Changes Associated with the Antibacterial Action of Silver Ions against Bovine Mastitis Pathogens

Seog-Jin Kang, Jae-Won Seol*, Tai-Young Hur[1], Young-Hun Jung, Changyong Choe and Sang-Youel Park*

National Institute of Animal Science, Rural Development Administration, Cheonan 330-801, Korea
**Center for Healthcare Technology Development, Bio-Safety Research Institute, College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Korea*

(Accepted: Dec 15, 2011)

Abstract : Silver has potent antibacterial activity against a variety of bacteria while maintaining low toxicity in mammalian cells. This study was conducted to investigate the possible mechanism underlying the bactericidal effects of silver ions against bovine mastitis pathogens using electron microscopy. We used two different bacterial strains, *Escherichia coli* and S*taphylococcus aureus*, which are primarily responsible for the majority of bovine mastitis cases. Interaction between the bacteria and silver ions (50 μg/mL, 2 hours) were studied using energy-filtering transmission electron microscopy (EFTEM). EFTEM images showed that *E. coli* and *S. aureus* cells treated with the silver ions had distorted plasma membranes, silver ions attached to the outer membranes, scattered electron-light material, and leakage of cell contents from disrupted cell membranes.

Key words **:** silver ion, mastitis, EFTEM, antibacterial activity.

Introduction

Silver has been long known to have strong inhibitory and bactericidal effects (3,9,10,28). Silver ion (Ag^+) solutions contain submicroscopic, positively-charged silver particles suspended in an aqueous medium. Silver ions acts as a powerful, broad-spectrum, anti-microbial agent and are known to kill over 650 different kinds of pathogens including bacteria, viruses, fungi, parasites, and molds (3,33). Furthermore, silver ions are also extensively used for food preservation, decontamination, and disinfection of medical supplies (7,12,21). Several studies have been conducted to examine the bactericidal effects of silver ions (18,19,25). It is generally believed that heavy metals react with SH groups, which leads to the inactivation of cellular proteins (11,19) and inhibition of bacterial oxygen metabolism.

Bovine mastitis is an inflammation of the mammary gland that often develops in response to intramammary bacterial infection and is the single most important cause of economic loss in the dairy industry (2). Pathogens that cause mastitis include bacteria and nonbacterial pathogens such as mycoplasmas, fungi, yeast, and *Chlamydia* (32). Although many bacteria can cause mastitis, *Staphylococcus aureus* and *Escherichia coli* are primarily responsible for the majority of bovine mastitis cases. Many researchers have explored the therapeutic effects of various agents against bovine mastitis (13,14,15,27,29).

[1]Corresponding author.
E-mail : tyohur@korea.kr

In particular, antibiotics have been critical in the fight against infectious diseases, such as mastitis, caused by bacteria and other microbes. However, disease-causing microbes that have become resistant to antibiotic drugs are an increasing threat to human health as well as in veterinary medicine (1). Thus, the interest to explore alternative, safe, and cost-effective antibacterial materials to cure animal disease is growing.

Several studies have reported the *in vitro* effects of silver on mammalian cells. Berger and colleagues reported that silver has potent antibacterial activity against a variety of bacteria while exhibiting low toxicity in mammalian cells (3,5). In our previous study, we also reported that silver ions do not have harmful effects on bovine mammary gland epithelial cells, and may inhibit the virulence of *S. aureus*-derived alpha-toxin in these cells (26). However, the mechanisms underlying the antimicrobial effects of silver are still not fully understood. The purpose of this study was to investigate the mechanisms underlying the bactericidal effects of silver ions against bovine mastitis pathogens such as *E. coli* and *S. aureus*. For this study, we used energy-filtering transmission electron microscopy (EFTEM) to observe morphologic changes in bacteria treated with silver ions.

Materials and Methods

Bacterial cultures

Two reference strains (*E. coli* O55 and *S. aureus* 305) were obtained from National Veterinary Research and Quarantine Service (Anyang, South Korea). The bacteria were sub-cul-

tured using Muller-Hinton agar plates (BD, USA). A large amount of microorganisms was scraped from the agar plate and used to inoculate test tubes containing Muller-Hinton broth (MHB; BD, USA). The MHB cultures were incubated at 37°C in ambient air until an optical density approximately equivalent to 1.0 McFarland standard (3×10^8 CFU/mL) was achieved.

Silver ion solution preparation

Silver ions were obtained from BioPlus Co. (Pohang, South Korea) (29). A colloidal silver (1000 μg/mL) solution was prepared from aqueous 0.01 M silver nitrate with various stabilizers (polyvinyl pyrrolidone and polyethylene). The pH of the solution was adjusted to 7.0 by adding sodium acetate. The silver ions were diluted (50 μg/mL) in pyrogen-free distilled water (Jungwei Pharma, South Korea). Two strains (*E. coli* and *S. aureus*) were incubated with silver ions (50 μg/mL) in MHB for 2 h.

Electron microscopy

EFTEM was used to examine the surface morphology of bacteria following silver ion treatment. Briefly, *E. coli* and *S. aureus* (2×10^7 cells) were incubated in MHB at 37°C for 2 h with or without silver ions (50 μg/mL). The cells were then centrifuged at 3,000 × g for 10 min. The samples were fixed with 2% glutaraldehyde and 1% osmium tetraoxide at room temperature. After eliminating the remaining glutaraldehyde and osmium tetraoxide, the samples were dehydrated with 30, 50, 70, 80, 95, and 100% alcohol. The fixed cells were embedded in Epon 812, and small blocks of bacteria in the Epon were cut using an ultramicrotome (Leica Ultracut UCT, Germany) with a diamond knife (Diatome, Switzerland). Ultrathin sections were then stained with uranyl acetate for 60 min followed by incubation with lead citrate for 5 min. The stained sections were examined using a Zeiss EM912Ω electron microscope (EFTEM) with a 120 kV accelerating voltage.

Results

Morphological changes in *E. coli* and *S. aureus* after silver ion treatment

To examine the morphological changes of bacterial cell caused by silver ion treatment, *E. coli* and *S. aureus* cells (2×10^7 cells) were incubated with or without silver ions (50 μg/mL) in MHB for 2 h. The morphological changes were observed by EFTEM. The untreated *E. coli* and *S. aureus* cells had normal morphologies such as a multilayered surfaces consisting of outer cell membranes, peptidoglycan layers in the periplasmic space, cytoplasmic membranes with normal electron density, and DNA localized at the center of the cells (Figs 1 and 2). However, all *E. coli* and *S. aureus* cells treated with silver ions showed many morphological changes including distortion of plasma membranes, silver ion attachment to the outer membranes, detachment of the cytoplasm from cell membranes, scattered electron-light materials, and even leakage of cell contents from disrupted cell membranes (Figs 3 and 4).

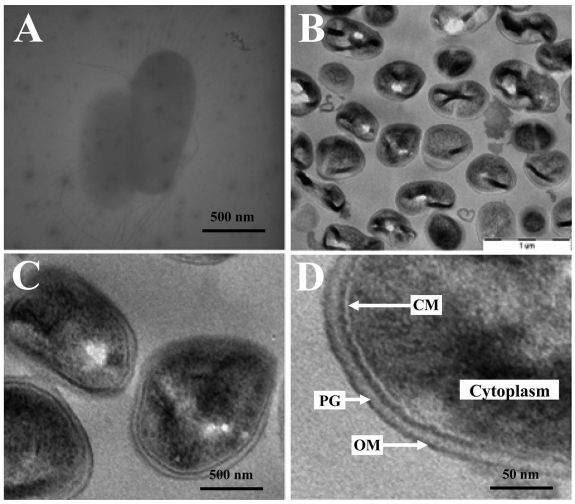

Fig 1. Internal structures in the untreated *E. coli* observed by transmission electron microscopy. Untreated *E. coli* (panels A and B); enlarged views of portion of panel B (panels C and D). OM = outer membrane, PG = peptidoglycan layer, CM = cytoplasmic membrane.

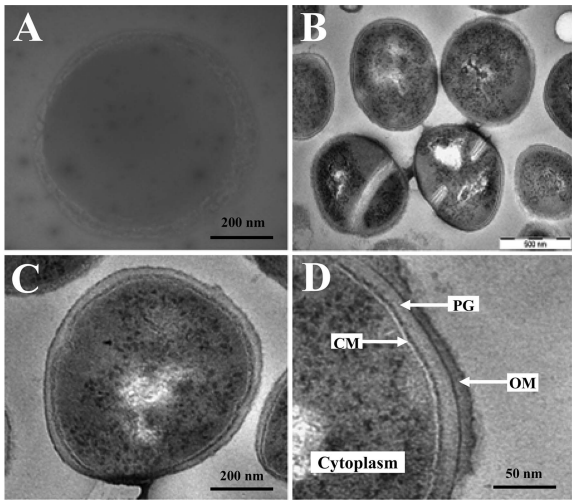

Fig 2. Internal structures of the untreated *S. aureus* observed by transmission electron microscopy. Untreated *S. aureus* (panels A and B); enlarged view of portion of panel B (panels C and D). OM = outer membrane, PG = peptidoglycan layer, CM = cytoplasmic membrane.

Discussion

Slawson *et al*. (1990) reported that silver binding may occur in two stages. The first stage is a rapid, reversible, and metabolically-independent surface binding. The second stage involves metabolically-dependent, irreversible, and intracellular accumulation of the silver ions. The bactericidal timeframe of silver ion indicated a close relationship between the

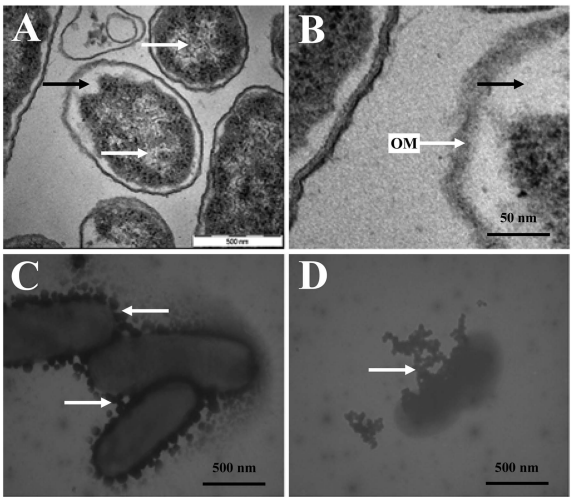

Fig 3. Internal structures of the silver ion-treated *E. coli* observed by transmission electron microscopy. *E. coli* were treated with 50 μg/mL of a silver ion solution for 2 h. (A) A remarkable electron-light region (white arrow) in the center of the cell and condensed DNA (white arrow) in the center of the electron-light region were observed. (B) Enlarged view of portion of panel A. A large gap between the cytoplasm membrane and the cell wall (black arrow) was observed. (C) Electron-dense granules were found around the cell wall (white arrow). (D) The cell wall was extensively damaged (white arrow).

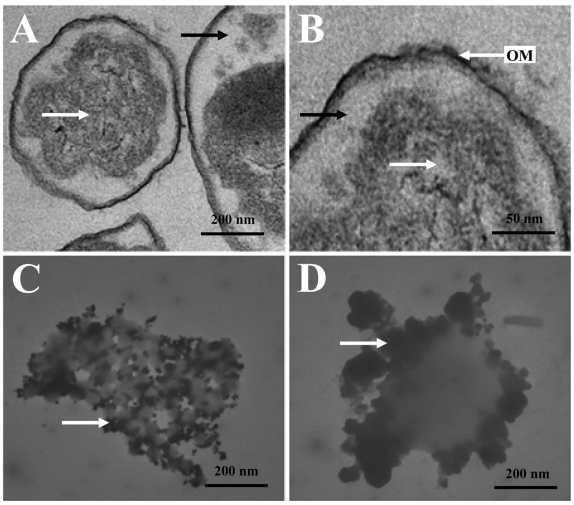

Fig 4. Internal structures of the silver ion-treated *S. aureus* observed by transmission electron microscopy. *S. aureus* were treated with 50 μg/mL of a silver ion solution for 2 h. (A) A remarkable electron-light region (white arrow) in the center of the cell and condensed DNA (white arrow) in the center of the electron-light region were observed. (B) Enlarged view of portion of panel A. A large gap was observed between the cytoplasm membrane and cell wall (black arrow). (C) Electron-dense granules were found around the cell wall (white arrow). (D) The cell wall was significantly damaged (white arrow).

silver concentration and killing time. Bactericidal action of the silver ions is characterized by the immediate onset of bacterial cell death, a short killing time, and a rapid rate of killing. Kumar and Munstedt (2005) showed that morphological changes in *E. coli* and *S. aureus* caused by silver ion treatment observed with SEM were probably caused by the attachment of silver ions to the outer cell surface. EF-TEM images further demonstrated the morphological changes that occurred following silver ion treatment in both *E. coli* and *S. aureus* cells. Detachment of cytoplasmic contents from the cell membrane, scattered electron light materials, disruption of cell wall, and eventual cell death were probably caused by the attachment of silver ions to cell surfaces and their diffusion into the cell. The small size and positive charges of the silver ions (19) probably allowed the ions to attach to bacterial cells, cross the cellular transportation barriers, and cause internal and external morphological changes in the bacteria.

In present study, many granules were observed around electron-light regions in the center of the cell. Silver ion granules were also detected inside and outside the cell membrane using EFTEM. It may be that attachment to and easy passage of silver into bacterial cells can cause internal and external distortions in both typical Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. Minor differences were also observed between the morphologies of *S. aureus* and *E. coli* treated with silver ions. In *E. coli*, the amount of electron-dense granules inside the cells as lower, and the electron-light region was comparatively darker than in *S. aureus*. These findings suggest that *E. coli* may possess a stronger defense system against silver ions.

Silver ions have a highly reactive moiety (6) and have a greater affinity for binding to proteins (24); this probably causes structural changes in bacteria. Electrostatic attraction between negatively-charged bacterial cells and positively-charged silver ion exist (19) which are crucial for explaining the antibacterial action of silver ions. The attachment of silver ions to cell proteins can significantly increase membrane permeability, leaving the bacterial cells incapable of properly regulating transport through their plasma membrane, ultimately leading to cell death. A similar effect was described by Klabunde *et al*. (2002) in *E. coli* treated with highly reactive metal oxide nanoparticles.

It is well known that the outer membrane of *E. coli* cells is predominantly constructed from tightly packed lipopolysaccharide (LPS) molecules, which form an effective permeability barrier (4). Wadu-Mesthrige *et al*. (2000) showed that attachment of heavy metals to cells may cause the formation of irregular-shaped pits in the outer membrane and changes membrane permeability. This is resulted by a progressive release of LPS molecules and membrane proteins. The silver ions may bind to and denature bacterial DNA and RNA, thereby inhibiting DNA replication (22). Feng *et al*. (2000) reported the formation of electron-light regions in the cytoplasm and condensation of DNA molecules in *E. coli* and *S. aureus* exposed to a silver nitrate solution. Nover *et al*. (1983) found that a heat stimulus harmful to the living cells can promote the localization of some low molecular weight proteins

around the nuclear region. Silver may elicit a type of shock to living cells different from that associated with heat treatment. However, it is possible that some of the proteins localized around the nuclear region of bacteria treated with silver ions seen by EFTEM in this study protect the DNA molecules. Rapid attachment and easy diffusion of silver ions into bacteria can cause the expansion of electron-light regions and the collapse of cell walls.

It is well known that the replication of DNA is only possible when the molecules are in a relax state (22). One probable reason to this is DNA condensation caused by silver ions that could lower the ability to the DNA to replicate (8). Furthermore, it was previously demonstrated that heavy metals can bind to thiol group when present inside the cell, thereby inhibiting the activity of various enzymes (34) and causing cellular death (19,20). Silver is a type of heavy metal that can induce the deposition of proteins in bacterial cells (8). Considering this, the small electron-dense granules observed outside the electron-light region could be deposits of silver and proteins.

The present results suggest that the bactericidal effects of the silver ion involved interaction of silver ion with the cytoplasm in the interior of the cell. Initially, the silver ions appear to penetrate through ion channels without causing damage to the cell membranes. Once inside the cells, great affinity to bind with proteins and small size of silver ion lead it to attach and penetrate the bacterial cells that resulted in morphological distortion, particularly formation of a large gap between the cytoplasm membrane and the cell wall. Silver is a kind of heavy metal that can cause the deposition of proteins in the cells. Therefore, the entrance of silver into bacterial cells may lead to the deposition of proteins in cells. In this study, we observed a remarkable scattered electron-light region in the center of the cell, condensed DNA in the center of the electron-light region and electron-dense granules around the cell wall. These processes seem to render the cell unable to sustain the membrane structures and results in the cell disruption.

Conclusion

The results of this study indicated that treatment with silver ions (50 μg/mL for 2 h) could kill bacteria that cause bovine mastitis (*E. coli* and *S. aureus*). Great affinity for proteins and small size enable the silver ions to attach to and penetrate the bacterial cells. This resulted in morphological changes, particularly formation of a large gap between the cytoplasm membrane and the cell wall. Furthermore, the entrance of silver ions into bacterial cells can cause DNA condensation and thereby inhibit DNA expression and replication resulted in disruption of cell wall and death of silver ion treated bacteria.

Acknowledgements

This work was carried out with the support of the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ9071162011)" Rural Development Administration, South Korea.

References

1. Bechert T, Steinrucke P, Guggenbichler JP. A new method for screening anti-infective biomaterials. Nature Med 2000; 6: 1053-1056.
2. Bennett RM, Christiansen K, Clifton-Hadley RS. Estimating the costs associated with endemic diseases of dairy cattle. J Dairy Res 1999; 66: 455-459.
3. Berger TJ, Spadaro JA, Bierman R, Chapin SE, Becker RO. Antifungal properties of electrically generated metallic ions. Antimicrob Agents Chemother 1976; 10: 856-860.
4. Berger TJ, Spadaro JA, Chapin SE, Becker RO. Electrically generated silver ions: quantitative effects on bacterial and mammalian cells. Antimicrob Agents Chemother 1976; 357-358.
5. Bramley AJ, Foster R. Effects of lysostaphin on staphylococcus aureus infections of the mouse mammary gland. Res Vet Sci 1990; 49: 120-121.
6. Coward JE, Carr HS, Rosenkranz HS. Silver sulfadiazine: effect on the ultrastructure of Pseudomonas aeruginosa. Antimicrob Agents Chemother 1973; 3: 621-624.
7. Dibrov P, Dzioba J, Gosink KK, Hase CC. Chemiosmotic mechanism of antimicrobial activity of Ag(+) in Vibrio cholerae. Antimicrob Agents Chemother 2002; 46: 2668-2670.
8. Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. J Biomed Mater Res 2000; 52: 662-668.
9. Fox CL. Silver sulphadiazine: a new topical therapy for Pseudomonas aeruginosa in burns. Arch Surg 1968; 96: 184-188.
10. Furr JR, Russell AD, Turner TD, Andrews A. Antimicrobial activity of Actisorb Plus, Actisorb and silver nitrate. J Hosp Infect 1994; 27: 201-208.
11. Holt KB, Bard AJ. Interaction of silver(I) ions with the respiratory chain of Escherichia coli: an electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag+. Biochemistry 2005; 44: 13214-13223.
12. Hotta M, Nakajima H, Yamamoto K, Aono M. Antibacterial temporary filling materials: the effect of adding various ratios of Ag-Zn-zeolite. J Oral Rehabil 1998; 25: 485-489.
13. Hu S, Concha C, Johannisson A, Meglia G, Waller KP. Effect of subcutaneous injection of ginseng on cows with subclinical Staphylococcus aureus mastitis. J Vet Med B Infect Dis Vet Public Health 2001; 48: 519-528.
14. Kai K, Komine K, Asai K, Kuroishi T et al. Antiinflammatory effects of intramammary infusions of glycyrrhizin in lactating cows with mastitis caused by coagulase-negative staphylococci. Am J Vet Res 2003; 64: 1213-1220.
15. Kawai K, Nagahata H, Lee NY, Anri A, Shimazaki K. Effect of infusing lactoferrin hydrolysate into bovine mammary glands with subclinical mastitis. Vet Res Commun 2003; 27: 539-548
16. Klabunde KJ, Koper OB, Klabunde JS et al. Nanoscale powders and formulations with biocidal activity toward spores and vegetative cells of bacillus species, viruses, and toxins.

Curr Microbiol 2002; 44: 49-55.
17. Kumar R, Munstedt H. Silver ion release from antimicrobial polyamide/silver composites. Biomaterials 2005; 26: 2081-2088.
18. Lansdown AB. Silver. I: its antibacterial properties and mechanism of action. J Wound Care 2002; 11: 125-130.
19. Liau SY, Read DC, Pugh WJ, Furr JR, Russell AD. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett Appl Microbiol 1997; 25: 279-283.
20. Lok CN, Ho CM, Chen R et al. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J Proteome Res 2006; 5: 916-924.
21. Mermel LA, Stolz SM, Maki DG. Surface antimicrobial activity of heparin-bonded and antiseptic-impregnated vascular catheters. J Infect Dis 1993; 167: 920-924.
22. Modak SM, Fox CL. Binding of silver sulfadiazine to the cellular components of Pseudomonas aeruginosa. Biochem Pharm 1973; 22: 2391-2404.
23. Nover L, Scharf KD, Neumann D. Formation of cytoplasmic heat shock granules in tomato cell cultures and leaves. Mol Cell Biol 1983; 3: 1648-1655.
24. Ovington LG Battling bacteria in wound care. Home Health Nurse 2001; 19: 622-630.
25. Russell AD, Hugo WB. Antimicrobial activity and action of silver. Prog Med Chem 1994; 31: 351-370.
26. Seol JW, Kang SJ, Park SY. Silver ion treatment of primary cultured bovine mammary gland epithelial cell (BMEC) damage from Staphylococcus aureus-derived α-toxin. Vet Res Commun 2010; 34: 33-42.
27. Seol JW, Hur TY, Jung YH, Kang SJ, Park SY. Application of Silver Ion for Clinical Mastitis in Holstein Cows. J Vet Clin 2010; 27: 246-251.
28. Seol JW, Hur TY, Jung YH, Kang SJ, Park SY. Evaluation of Bactericidal Capacity of Silver Ion against Bovine Mastitis Pathogens. J Vet Clin 2010; 27:252-256.
29. Shin HS, Yang HJ, Kim SB, Lee MS. Mechanism of growth of colloidal silver nanoparticles stabilized by polyvinyl pyrrolidone in γ-irradiated silver nitrate solution. J Collo Interf Sci 2004; 274: 89-94
30. Slawson RM, Lee H, Trevors JT. Bacterial interactions with silver. Biol Metals 1990; 3: 151-154.
31. Wadu-Mesthrige K, Amro NA, Liu GY. Immobilization of proteins on self-assembled monolayers. Scanning 2000; 22: 380-388.
32. Watts DH, Eschenbach DA. Treatment of Chlamydia, Mycoplasma, and group B streptococcal infections. Clin Obstet Gynecol 1988; 31: 435-452.
33. Wright JB, Lam K, Hansen D, Burrell RE. Efficacy of topical silver against fungal burn wound pathogens. Am J Infect Cont. 1999; 27: 344-350.
34. Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ. Effect of pooled human cerebrospinal fluid on the postantibiotic effects of cefotaxime, ciprofloxacin, and gentamicin against Escherichia coli. Antimicrob Agents Chemother 1992; 36: 1136-1139.

은 이온의 항균효과에 대한 소 유방염 원인균의 형태학적 변화

강석진 · 설재원* · 허태영[1] · 정영훈 · 최창용 · 박상열*

농촌진흥청 국립축산과학원, *헬스케어 기술개발 사업단, 전북대학교 수의과대학 생체안전성 연구소

요 약 : 은 이온(silver ion)은 동물세포에 낮은 독성을 가지면서도 다양한 종류의 세균들에 대해 강력한 항균력을 가지고 있다. 본 연구에서는 젖소 유방염 원인균들에 대한 은 이온의 항균효과를 전자현미경(TEM)을 통해 조사하였다. 본 실험에 사용된 균주는, 젖소 유방염의 주요 원인균인 *Escherichia coli* 와 *Staphylococcus aureus*를 사용하였다. 유방염 원인균에 대한 은 이온의 항균효과를 관찰하기 위해 50 μg/mL의 은 이온을 2시간 동안 세균에 노출시킨 후 전자현미경(Energy-Filtering Transmission Electron Microscopy, EFTEM) 을 이용하여 은 이온에 의한 세균의 형태 변화를 확인하였다. 은 이온 처리 후 전자현미경 촬영 결과 *E coli* 와 *S aureus*의 세포막이 변형되고, 세포 외부에 침착된 은 이온과, electron-light 모습으로 세포내 분산된 은 이온을 관찰 할 수 있었으며, 결과적으로 *E coli* 와 *S aureus* 의 세포막이 파괴되어, 세포내용물이 외부에 누출됨으로써 세균이 사멸되는 것을 확인하였다.

주요어 : 은 이온, 유방염, 전자현미경, 항균작용