Abstract: CuraVAC Ag, a product that delivers negative pressure wound therapy through a polyurethane foam dressing, contains silver nanoparticles, which, when moistened with water, release silver ions onto a wound surface. The *in vitro* antimicrobial action of silver can destroy both gram-positive and gram-negative bacteria, as well as methicillin-resistant *Staphylococcus aureus* (MRSA). The purpose of this study was to assess the efficacy and *in vivo* outcomes of using the product. Methods. Thirty-six female Sprague-Dawley white rats, 8-weeks old and 250 g - 300 g in weight, were used. The experimental product was prepared using a vacuum-assisted closure (VAC) kit and coating it using the silver nanoparticles. For the control group, a 10% povidone-iodine solution was applied. Results. All groups showed decreases in wound area over time, in the order CuraVAC Ag (group A) > CuraVAC (group B) > control (group C). On the third, fifth, and seventh days, wound healing efficacy scores increased in both group A and group C. Groups A and B showed more rapid decreases than group C in bacterial culture from wounds. Conclusion. CuraVAC Ag may be useful for treatment of wounds infected with bacteria.

Key words: nanosilver, vacuum-assisted closure, MRSA, rat
widely used polymer for medical applications, and was chosen as the base material. The purpose of this study was to assess the efficacy and in vivo outcomes of using the product.

Materials

The synthesis of silver nanoparticles is now well-understood, and several methods have been developed that offer good control over particle size and morphology. Colloidal silver nanoparticles were synthesized by reducing silver nitrate in water-soluble chitosan solution, which does not involve the use of any toxic chemicals. All reagents used in this study are considered to be nontoxic.

In preparation for synthesis of the colloidal silver nanoparticle, a silver nitrate (AgNO₃), of analytical purity (Sigma-Aldrich, St. Louis, MO) and water-soluble chitosan (Kitto Life Co, Ltd, Seoul, Korea) were purchased.

In a typical synthesis, AgNO₃ was first dissolved in distilled water and alcohol at room temperature to obtain 1.0% w/w solution. The volume ratio of water to alcohol was 70:30. Then, AgNO₃ solution was slowly dripped into water-soluble chitosan solution with ultrasonication until dripping was complete (typically 5 minutes). Then, ultrasonic mixing was replaced by mechanical mixing for 30 minutes to allow crystal formation. The solution was dialyzed against 1.0 L of distilled water using a dialysis tube with a molecular cutoff of 3,500 g/mol. A gray-colored solution of silver was thus obtained.

Polyurethane foam was dipped in the silver nanoparticle solution, with stirring at room temperature, followed by air drying overnight. The silver nanoparticle-coated foam dressing was then dipped in 1 M tripolyphosphate (TPP) solution and air dried overnight.

Characterization of the Silver Nanoparticles

Particle size and distribution measurement. Particle size and size distribution of the silver nanoparticles were investigated by dynamic light scattering (DLS). Dynamic light scattering measurements were carried out using an ELS-8000 electrophoretic LS spectrophotometer (Otsuka Electronics Co Ltd, Osaka, Japan) equipped with an argon laser operating at 632.8 nm with a fixed scattering angle of 90 degrees. The silver nanoparticle concentration in distilled water of the samples was 1 mg/mL.

Transmission electron microscopy. Silver nanoparticle morphology was examined with a JEOL JEM-2000 FX-II transmission electron microscope (TEM). A drop of each nanoparticle suspension was placed on a carbon film coated on a copper grid for TEM.

Characterization of Medical-Grade Polyurethane with Silver Nanoparticles

Fourier transform infrared spectroscopy. Fourier transform infrared spectra (FTIR) were recorded on a potassium bromide (KBr) disc in dry air at room temperature. The KBr disc was prepared by mechanically blending the silver nanoparticles (3 mg) with 300 mg of KBr for 10 minutes, and the KBr disc was obtained from a 200 mg aliquot of the mixed powder.

Scanning electron microscopy. Micrographs of the samples were taken using a field emission scanning electron microscope (FESEM) (JSM-6700F, JEOL, Tokyo, Japan). Prior to observation, all samples were coated with gold and scanned at an accelerating voltage of 15kV.

Antimicrobial test. Antimicrobial efficacy testing was performed according to the KS J 4206 method. Briefly, a 1 cm × 1 cm × 1 cm sample was cut into 3 specimen cubes. The specimens were then placed into 2 mL of ~10⁵ CFU/mL Staphylococcus aureus ATCC 6538, or Escherichia coli suspensions, and incubated at 37°C. At 1 hour, 6 hours, 12 hours, and 24 hours, the levels of viability of both microorganisms were determined by ultraviolet spectrophotometry (UV-1601, Shimazu, Kyoto, Japan).

Experimental animals and protocol. Thirty-six female Sprague-Dawley white rats, 8-weeks old and 250 g - 300 g in weight, were used. In the laboratory, the rats were raised using standard fodder and water, and the experiment was carried out after a 1-week adjustment period. Animals were maintained under a standardized light schedule and housed in individually ventilated cages. All animal studies were approved by the author’s institutional committee for the care and use of animals in research and education. Animals were treated in accordance with the guidelines issued by the National Institutes of Health Office of Laboratory Animal Welfare.

Experimental Strain. Methicillin-resistant Staphylococcus aureus ATCC 43300 was used as the standard strain, at a turbidity of 0.5 McFarland units.

Experimental Product. The experimental product was prepared by coating polyurethane foam dressing from a vacuum-assisted closure (VAC) kit (CuraVAC, Daewoong Pharm Co, Ltd, Seoul, Korea) with silver nanoparticles (CuraVAC Ag, Daewoong Pharm Co, Ltd). For the control group (group C), a 10% povidone-io-
dine solution (Betadine, Sungkwang Pharm Co, Ltd, Bucheon, Korea) was applied.

Methods

The rats were shaved after administration of an intramuscular injection into the inside of the right leg of 100 µL of the animal anesthetics tiletamine hypochloride and zolazepam hypochloride (Zoletil®, Virbac, Carros Cedex, France), and Xylazine (Rompun, Bayer Korea, Seoul, Korea), mixed at a ratio of 4:1. The hair on the rats’ backs was removed, and the backs disinfected with 70% ethanol. A full-thickness rodent skin wound 1 cm × 1 cm was created. Methicillin-resistant Staphylococcus aureus was administered into the formed wound by dripping 0.1 mL of a 0.5 McFarland unit turbidity suspension, then the wounds were fully covered with 2 cm × 2 cm petroleum jelly gauze. To prevent the wounds from drying, the uppermost section of the gauze was sealed using a transparent waterproof film (Opsite Flexifix Smith & Nephew, London, UK) and fixed with tape.

After infection, the 36 rats were divided into 3 groups of 12 rats each. Group A was treated using the silver nanoparticle-coated VAC kit; group B was treated with the standard VAC kit; and group C was treated using a 10% povidone-iodine solution. A 125 mm Hg of pressure was applied to the foam dressing with wound treatment in 2-day intervals. During these experiments, rats were housed individually and during the observation period, feed and water were available ad libitum.

After treatment on the third, fifth, and seventh days, a disinfected transparent film was placed over the wound and marked the wound boundary. The film was then scanned and wound area measured using a digital image analysis tool (OlyDbg, alpha 4, version 2.01, Microsoft Corp, Redmond, WA). The measured area was calculated in cm² with the surface area on the first day after the wounds were prepared deemed to be 100%, and changes compared to that area.

On the third, fifth, and seventh days after the wounds had been prepared, 4 rats in each group were sacrificed, and tissue near the wounds and the wound itself were collected. Tissues were fixed in 10% formalin solution for >6 hours and prepared for hematoxylin-eosin staining to allow observation of reepithelization, granulation, necrosis, and inflammation levels. Reepithelization and granulation levels were scored from 0 to 3 and the results were digitized. When reepithelization and granulation had not been achieved, the result was recorded as 0. When reepithelization and granulation were not achieved, and the defect reached more than two-thirds of the wound surface area, the result was scored as 1. When the defect covered one-third to two-thirds of the wound surface area, the result was scored as 2, and when it covered less than one-third, the result was scored as 3.

Necrosis and inflammation levels were also scored from 0 to -3. The result was scored as 0 when no necrosis or inflammation was found, or when the cells were partially inflammatory, without forming a cluster. The result was scored as -1 when inflammatory cells partially appeared in a cell form, but without forming a cluster. The result was -2 when the cluster was not obvious, but presented as a mixed state of small clusters and inflammatory cells. The result was scored as -3 when a large cluster was formed.

The scores for reepithelization, granulation tissue, normal stratum corneum, and stratum were summed, to give totals from -6 to 6. The larger the value, the better the wound healing effect.

On the third, fifth, and seventh days after wounds were prepared, 4 animals per group were sacrificed and tissues of the open wounds were sampled by a biopsy for bacterial culture. Of the biopsied tissues, 1 g was treated using a tissue homogenizer, and 0.5 mL of a phosphate-buffered saline was added before centrifugation for 5 minutes at 1,500 rpm to ‘clean’ the sample. This cleaning process was repeated once. The cleared suspension, 0.001 mL was inoculated onto blood agar medium and cultivated for 24 hours at 35°C. The number of colonies was then counted.

Statistical Analysis

Data are expressed as means ± standard deviations (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Tukey’s test, to correct for multiple comparisons. A level of significance of 5% was set with regard to differences between group means. All hypothesis tests were 2-sided. All statistical

**Key Points**

- After infection, the 36 rats were divided into 3 groups of 12.
- Group A was treated using the silver nanoparticle-coated VAC kit; group B was treated with the standard VAC kit; and group C was treated using a 10% povidone-iodine solution.
- 125 mm Hg of pressure was applied to foam dressing with wound treatment in 2-day intervals.
analyses were conducted with the SAS 9.2 (SAS Institute Inc, Cary, NC).

Results
Characterization of the silver nanoparticles. The size of the silver nanoparticles was analyzed using DLS and TEM. The mean diameter of the particles was 100 nm ± 56.3 nm (Figure 1). The size distribution showed a unimodal form without large and/or small particles. Transmission electron microscopy photographs of silver nanoparticles showed an almost spherical shape and a size range of ~50 nm (Figure 2). This value was close to the average diameter determined by DLS and TEM.

Characterization of the foam dressing coating. Coating of the foam dressing was verified using FTIR, and the FTIR spectrum of the foam dressing indicated the coating. After coating, peaks at 1539 cm⁻¹ and 1635 cm⁻¹ appeared. These peaks are characteristic of chitosan, demonstrating that the PU was coated successfully (data not shown).

Morphology of the foam dressing coating. Scanning electron microscopy images of the sample showed the chitosan coating (Figure 3). The coating thickness was ~3 µm 5 µm. Images demonstrated that the PU coating had an average pore size of 400 µm - 600 µm.

Antimicrobial test. The antimicrobial efficacy of the coated foam dressing was demonstrated by a 99.9% reduction in viable S. aureus and E. coli numbers after 1 hour, 6 hours, 12 hours, 24 hours, and 48 hours of exposure to the foam (Figure 4). Antimicrobial efficacy
was thus maintained over the maximum recommended dressing change interval of 48 hours.

All groups showed decreases in wound area over time, in the following order: group A > group B > group C. Groups A and B showed more rapid decreases in wound area, and at the final wound area measurement on the seventh day, the decreases were markedly larger than in group C. The difference among the 3 groups was statistically significant (2-way ANOVA, \( P = 0.0459 \)) (Figure 5).

Daily observations showed reepithelization and granulation tissue, and an increase in inflammatory cell infiltration (Figure 6). On the third, fifth, and seventh days, wound healing efficacy scores increased in both group A and group C (Figure 7).

The numbers of viable bacteria decreased in all groups, but groups A and B showed more rapid decreases. However, on the seventh day, group A indicated fewer bacteria than group B (Figure 8).

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Discussion

Morykwas et al\textsuperscript{8} published a seminal paper about VAC in 1997, and the technique is now known to be effective for wound healing. With this method, sound pressure is used to remove edema and body fluid from wound tissues and increase the formation of blood stream and granulation tissues, through mechanical loading of the VAC system, which eventually removes bacteria from the wound.\textsuperscript{8-10}

In the present study, VAC foam was coated with water-soluble chitosan and silver nanoparticles, uniformly mixed with distilled water and an ethane solution using ultrasound. The uniformly mixed solution was purified using dialysis and the remains were removed to produce gray-colored silver nanoparticles. The solution was spray-coated on VAC foam and dried. Then, the coated solution was cross-linked to the VAC foam and its antibacterial efficacy assessed. Vacuum-assisted closure foam exhibited a pore size of ~ 400 µm -600 µm.\textsuperscript{7}

Negative pressure wound therapy has been widely applied to acute and chronic wounds including diabetic foot ulcers, pressure ulcers, and wound dehiscence after surgery.

Group A, treated with the product containing silver, and group B, which was treated with the normal PU foam, both showed better antimicrobial efficacy than that of group C, the control group treated with 10\% povidone-iodine solution. Additionally, group A exhibited a greater efficacy than group B. It appears the inclusion of ionized silver induces sterilization with regard to MRSA dripped into wounds. Additionally, in terms of changes in the wounds, group A and group B showed better sterilization efficacies than group C. The wound-healing efficacy improved upon inclusion of silver in the foam. Further, coating of the foam with silver was not problematic.

However, the histological scores of the wound tissues showed no significant differences. It appears that frequent changes of the dressing every other day stimulated the wounds and thus disturbed wound healing. In the process of changing the PU foam, granulation tissues were removed that had attached to the foam. This was likely because the granulation tissues grew into the pores of the foam. This problem should be addressed in future studies.

It has been reported that silver ions at a low concentration rarely cause skin coloration or cytotoxicity issues. However, experiments conducted with human epithelial cells showed that a silver-containing dressing was toxic, and there have been few studies on its long-term effects.\textsuperscript{11,12} In this study, the authors detected no coloration or cytotoxicity. However, the amount of free silver was not measured, so the authors do not know how much silver was deposited into the tissues; thus, it was impossible to predict whether coloration of wounds or other tissues would occur. Further studies are necessary to examine loss of silver from the foam, stability, and cytotoxicity.

As noted above, silver ions act on bacteria in several ways and at very low concentrations, so there is a possibility, albeit remote, that bacteria will develop resistance. Thus, silver may be useful as a new antimicrobial. Additionally, silver particles have excellent reduction and antitoxic efficacies, and have the advantage of being readily synthesized and absorbed onto cell surfaces.\textsuperscript{13,14} Thus, silver is expected to be further

Figure 7. Mean wound-healing grade by day and dressing groups.

Figure 8. Mean number of colonies (x105 CFU/ml) by day and dressing groups.
used in various fields, including dressings. This study also demonstrated that silver-facilitated wound healing. However, there have been few studies on the stability of silver and its long-term effects on the human body. Thus, it is necessary to confirm various preliminary studies, research the possibility of adverse effects, and conduct long-term and clinical studies.

Conclusions
Vacuum-assisted closure foam, as currently used, is known to be effective in wound healing, and this study showed that VAC had better results than a dressing used in conjunction with a 10% povidone-iodine solution. Additionally, when a silver-coated foam dressing was used in conjunction with VAC, the wound area-reduction and antibacterial efficacy increased further. Silver-coating the surface of VAC foam using ultrasound was not problematic. Thus, CureVAC Ag, a product formed by coating a VAC kit with silver nanoparticles, may be useful for treatment of wounds infected with bacteria.

Acknowledgements
This research was supported by the Basic Science Research Program through the National Research Foundation of Korea, funded by the Ministry of Education, Science, and Technology (2011-0013275).

References