Silver has been used for its antimicrobial properties for a long time; even ancient Greeks applied silver chips or granules to wounds of injured soldiers to prevent infection. Ingestible silver in different soluble preparations has also been used, with the intention to cure many diseases. Silver nitrate and silver sulphadiazine have been used extensively in the care of burns for many years, with few questions asked. However, in 1977, Bridges and Lowbury raised a question about possible microbial resistance to silver, something that has been brought to the fore today when drug resistance is discussed.

With the extended use of different silver dressings, additional side effects such as staining of the skin, reduced wound healing, and increased wound pain of patients treated repeatedly with silver dressings have been reported and described. These phenomena have rightfully caught the attention of researchers. For example, Trop presented a case in which raised liver

Abstract: Silver is commonly used in wound dressings and topical formulations to assist in the management of wounds that are infected or at risk of becoming infected. They provide potent broad-spectrum antimicrobial activity, but should not cause sustained staining of the skin, dermal or systemic accumulation of silver, or discomfort to the patient. However, clinicians and healthcare personnel have been concerned about topical staining of the skin and complaints of additional pain from patients treated with certain silver dressings. Some delay in re-epithelialization has also been noticed and reported. The reasons for this are not clear, and the authors believed further study regarding the possible effects of silver accumulation and silver dressings’ effect on re-epithelialization was required. The authors studied possible silver accumulation and re-epithelialization in normal human dermal skin. The results showed that most of the dressings or treatments discolored the wound surface and that there was a dermal accumulation of what were assumed to be silver particles. Varying grades of accumulation were found in deep dermal tissue, particularly around blood vessels, depending on the dressing used. The results also indicated that all of the tested products delayed re-epithelialization in this model.
enzymes and argyria-like symptoms were noted when a patient with 30% total body surface area (TBSA) burns was treated with Acticoat™ (Smith & Nephew, Fort Lauderdale, Fla). Poon and Burd described silver to be highly cytotoxic to keratinocytes and suggestions were made that consideration of the cytotoxic effects of silver and silver-based products should be taken into account when deciding on what dressings to use, particularly when using cultured keratinocytes in situ. This is playing an increasing role in contemporary care of wounds and burns. Vlachou et al performed studies on systemic silver absorption in patients using Acticoat and showed that only small quantities of silver were absorbed systemically, which led to their recommendations for using Acticoat in the treatment of burns. Innes et al reported that donor sites treated with Acticoat needed more time to heal compared with the control wounds treated with Allevyn™ (Smith & Nephew, Fort Lauderdale, Fla). The donor sites treated with Acticoat had worse scarring by 2 months and the authors stated that their results did not support its use on donor sites. A blackening of the wound and surrounding skin that was visible when treating a wound with products containing silver is often merely transient binding of silver to wound debris and epidermal cells, which will be shed as the wound heals. However, silver particles from topical applications can penetrate human tissues and be found systemically, though in small quantities. Despite this, we could find few reports regarding the accumulation of silver in dermal tissue, which theoretically would be the tissue subjected to the highest concentrations.

Any study of a physiological process demands a model that resembles the conditions seen in vivo, but in-vivo wounds are difficult to standardize, as factors that affect wound healing vary among individuals—eg, age, nutritional state, and the presence of infections. In-vitro wound models that consist of either a cell monolayer or cells cultured in 3-dimensional matrices give repeatable results, but do not completely resemble the complexity of the healing process that is seen in human skin. The in-vitro model, developed by Emanuelsson and Kratz, in which the re-epithelialization of the wounds was followed histologically throughout the time of incubation (14 days) by fixing and staining wounds every second day. After 14 days of incubation, the viability of the cells in the epidermis and dermis was confirmed by isolation and culture in vitro. The wounds incubated in 10% fetal calf serum were shown to heal after 7 days, whereas wounds incubated in 2% serum did not show any signs of re-epithelization. However, both epidermal and dermal cells from wounds incubated in 2% serum were shown to be viable after 2 weeks of incubation. Thus, wounds incubated in 2% fetal calf serum could be compared to a chronic nonhealing wound.

The present study concentrates on accumulation of silver in the dermis. Additionally, re-epithelialization was studied and graded to estimate whether the different dressings affected the healing of the wounds in any way.

Materials and Methods

Wound model. Normal human skin from surgical waste (abdominoplasty) was transferred sterile to the laboratory in gauze soaked in physiological saline. The skin was rinsed twice in phosphate buffered saline supplemented with antibiotics and mycotics (penicillin 50 IU/mL, streptomycin 50 g/mL). Subcutaneous fat was removed with scissors and circular discs of skin were made from the remaining tissue (dermis and epidermis) using an 8-mm diameter skin biopsy punch (Kai Medical, Solingen, Germany). A dermal wound was made in the center of each disc roughly 1-mm deep using a 3-mm diameter skin biopsy punch. The discs (3 skin discs/group) were subsequently transferred to Transwell cell culture inserts (pore size 0.4 µm) in 6-well plates (BD Falcon, Stockholm, Sweden). Cell culture medium was added to the outer wells, leaving the discs in the air and the liquid interface in the inner cell culture insert, to facilitate the application of the viscous dressings—silver sulphadiazine (Flamazine®, Smith & Nephew, Fort Lauderdale, Fla) and silver nitrate—to prevent the dressings from floating off the discs (Illustration 1). Under sterile conditions, pieces of Acticoat, Aquacel® Ag (Convatec, Princeton, NJ), PolyMem® Silver™ (Ferris Mfg Corp, Burr Ridge, Ill), SilvA sorb® (Medline, Mundelein, Ill), and Silverlon® (Argentum Medical, Chicago, Ill), roughly 8-mm in diameter, were cut and applied to the wounds.

A thin layer of Flamazine was applied with a sterile cotton swab, and a drop of silver nitrate was dripped on to the wound. The dressings were moistened with sterile water (as recommended by the dressing manufacturers) and the wounds were incubated at 37°C (5% carbon dioxide and 95% humidity) for 14 days before sampling. All silver dressings used were applied to the wounds and changed according to the manufacturers’ directions (Table 1). The culture media was changed every second day throughout the study. Skin discs were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with antibiotics and mycotics (penicillin 50
IU/mL and streptomycin 50 µg/mL), and with 10% (positive control and treatment groups), or 2% (negative controls) fetal calf serum, respectively. All culture mediums were changed every second day throughout the study.

### Sampling
After 14 days of culture, the wounds were fixed in 4% neutral buffered formaldehyde, dehydrated through a xylene-ethanol series and embedded in paraffin for later histological examination.

### Histology
Cross sections of the paraffin-embedded wounds (7-µm thick) were stained using hematoxylin and eosin, and re-epithelialization was measured with an Olympus BX41 microscope. Images were captured with an Olympus DP70 CCD camera.

### Immunostaining for von Willebrand factor
Immunohistochemical staining was used to verify the structures in the tissue where the deposits were found. Non-specific protein binding was blocked with 2% normal horse serum in phosphate buffered saline (PBS) for 20 minutes. Immunohistochemical analysis for endothelial cells was performed using a mouse monoclonal antibody raised against the glycoprotein von Willebrand factor (Dako M0616, DakoCytomation, Glostrup, Denmark). The sections were incubated with primary antiserum diluted in the ratio of 1:25 in PBS for 30 minutes at room temperature, rinsed in PBS, and incubated with a biotinylated secondary antibody (2 g/mL) for 30 minutes. After washing, the bound antibody was localized with an avidin-peroxidase Vectastain® VIP-kit (Vector Laboratories Inc., Burlingame, Calif.) with hydrogen peroxide as peroxidase substrate. Native skin served as positive control for the immunohistochemistry; negative control was omission of the primary antibody. Sections were examined using an Olympus BX41 microscope, and images were captured with an Olympus DP70 CCD camera.

### Results

#### Re-epithelialization
All groups were stained using hematoxylin and eosin. Wound re-epithelialization was studied. Wounds in the positive control groups (incubated in 10% FCS) showed complete re-epithelialization after 14 days of incubation (Figure 1A). Wounds in the negative control groups (incubated in 2% FCS) showed no signs of re-epithelialization (Figure 1B). Wounds treated with Acticoat, Silverlon, SilvaSorb, or silver nitrate did not show any signs of re-epithelialization (Figure 1C–G). Wounds treated with Flamazine and PolyMem Silver showed signs of re-epithelialization, such as buds of epithelial cells at the wound margins, and a small tongue of epithelial cells extending toward the center of the wounds (Figure 2A). Wounds treated with Aquacel Ag showed a tongue of epithelial cells extending over the wound bed, representing active (but not yet complete) re-epithelialization (Figure 2B).

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**Table 1.** Treatment regimens used in the study. All groups were cultured for 14 days. Skin discs/wounds were cultured in Dulbecco’s modified Eagle’s medium supplemented with antibiotics/mycotics (penicillin 50 U/mL and streptomycin 50 µg/mL) and with 10% (positive control and treatment groups) or 2% (negative controls) fetal calf serum, respectively. All culture mediums were changed every second day throughout the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment interval (dressing changes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>No treatment</td>
</tr>
<tr>
<td>Negative control</td>
<td>No treatment</td>
</tr>
<tr>
<td>Acticoat</td>
<td>Every second day</td>
</tr>
<tr>
<td>Aquacel Ag</td>
<td>Every second day</td>
</tr>
<tr>
<td>Silver sulphadiazine (Flamazine)</td>
<td>Every day</td>
</tr>
<tr>
<td>PolyMem Silver</td>
<td>Every seventh day</td>
</tr>
<tr>
<td>SilvaSorb</td>
<td>Every second day</td>
</tr>
<tr>
<td>Silverlon</td>
<td>Every second day</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>Every day</td>
</tr>
</tbody>
</table>

**Illustration 1.** A schematic of how the wounds were created in human skin from surgical waste (1–4); placed in a cell culture insert in a 6-well plate (5); and dressed with silver dressing (6) before incubation (7).
Silver deposition. No particles, discoloration, or deposits were seen in any of the control groups at any level or structure (Figure 3A, B).

Acticoat. Acticoat is a dressing consisting of an absorbent rayon/polyester core, covered on both sides by silver plated, high-density polyethylene. The silver in the dressing has a nanocrystalline structure with about 30 to 50 silver atoms in each crystal.

Findings. A black discoloration was seen in the epidermal layer. Black or gray deposits were also seen in cells at the wound margins and around blood vessels in the dermal tissue. Wounds treated with Acticoat showed more deposits than all other treatment groups (Figure 4A, B).

Aquacel Ag. Aquacel Ag is a dressing consisting of sodium carboxymethylcellulose fibres in the form of a fleece, containing 1.2% silver in ionic form. When in contact with (wound) fluids the Aquacel Ag dressing forms a
hydrophilic gel that traps microbes and releases silver ions through the interaction with sodium ions.

**Findings.** There was no discoloration in the keratinocyte layer in the wounds treated with this product. Particles and some aggregate-containing cells were seen at the wound margins together with particles aggregated adjacent to blood vessels throughout the dermis. Wounds treated with Aquacel Ag had moderate deposits (Figure 5A, B).

**Flamazine.** Flamazine consists of 1.0% micronized silver sulphadiazine in a hydrophilic cream base. Sulphadiazine, as well as silver, provides an antimicrobial effect.

**Findings.** Slight discoloration of the keratinocyte layer was seen in these wounds. Cells with deposits were seen at the wound margins, throughout the wound, and also assembled around some blood vessels. Wounds treated with Flamazine had moderate deposits (Figure 6A, B).

**PolyMem Silver.** PolyMem Silver is composed of a polyurethane matrix on a semi-permeable thin film backing that promotes oxygen and vapor exchange. The dressing contains silver in a nanocrystalline form.

**Findings.** There was no discoloration in the keratinocyte layer in the PolyMem Silver group. There were no deposits in these wounds at any structure or level (Figure 7A, B).

**SilvaSorb.** SilvaSorb is composed of a synthetic polyacrylate, with a highly absorbent MicroLattice® matrix containing stabilized silver. There was no discoloration in the keratinocyte layer in this group.

**Findings.** There were no particles deposited around blood vessels and no deposits in these wounds at any structure or level (Figure 8A, B).

**Silverlon.** Silverlon is a 3-dimensional mesh of nylon fibers, plated with silver, through the use of an autocatalytic reduction-oxidation plating technology, resulting in silver released into the wound fluid in ionic form (Ag+) and is not colloidal (Ag0).

**Findings.** There was no discoloration in the keratinocyte layer. However, cells with black or gray deposits were seen at the wound margins and throughout the dermis. Particles had also aggregated around blood vessels.
Wounds treated with Silverlon had moderate deposits compared to groups treated with other products (Figure 9A, B).

**Silver nitrate.** The effects of silver nitrate probably result from silver ions readily combining with sulfhydryl-, carboxyl-, phosphate-, amino-, and other biologically important chemical groups.

**Findings.** There was no discoloration in the keratinocyte layer of wounds treated with silver nitrate, but a thick layer of black particles covered the surfaces of the wounds. Cells containing black or gray aggregates were seen at the wound margins, throughout the wound bed, and also around a few blood vessels in the dermis. These wounds also showed moderate deposits (Figure 10A, C).

**Immunohistochemistry.** Immunoassays that targeted von Willebrand factor were used to verify that the circular structures surrounded by black or gray shadows were in fact blood vessels. Cross-sections from the control groups showed staining for von Willebrand factor in the endothelial cells in native skin incubated with primary antibody (Figure 11A) and no staining of the controls with the omission of the primary antibody (Figure 11B). Wounds from all groups treated with silver dressings, except for PolyMem Silver and SilvaSorb, had similar circular deposits around structures in the dermal layers (Figure 12A, B).

**Discussion**

The Western world has used the silver ion as a topical antimicrobial agent in burn care for many years. Klasen dated the use of silver back several hundred years. The toxicity of silver ions has not attracted much attention over the years, even though there are studies about it. Bridges and Lowbury suggest that silver sulphadiazine induced severe chronic inflammation in burns, but Vlachou et al state Acticoat is safe to use and should remain a standard part of the treatment at their clinic.

In the present study, black discoloration or particle deposits (or both) were seen throughout the tissue in all
wounds treated with products containing silver, except for PolyMem Silver and SilvaSorb. At the wound margin and around blood vessels, there were clusters of cells that contained black particles and dark deposits around structures that were confirmed to be blood vessels through immunostaining for von Willebrand factor. However, not all blood vessels had deposits, and the number of particles around individual blood vessels varied. Sets of controls, incubated without any products containing silver, showed no signs of deposits or discoloration, which eliminates the medium and the wounds themselves as the source of discoloration. This leads to the conclusion that it is the treatment with products containing silver that results in black deposits throughout the tissue, not any surrounding factor. It seems unlikely to be anything other than silver, as all products have been manufactured in different ways and contain different materials and substances; silver is the only common denominator. Therefore, there is a strong likelihood that the discoloration and deposits in the wounds and surrounding tissue consist of silver that was released during treatment. However, not all products tested in this study left deposits in the tissues. Groups treated with PolyMem Silver and SilvaSorb showed no signs of discoloration or deposits, whereas all other products caused varying degrees of deposits near blood vessels and wound margins. Acticoat caused the most deposits, and was the only dressing to cause black discoloration of the keratinocyte layer. Aquacel Ag caused the second most deposits, followed by Silverlon, Flamazine, and SilvaSorb. The results also indicate that all products tested delayed wound healing. The wounds that were treated with Acticoat, Silverlon, and SilvaSorb showed no signs of re-epithelialization, and of all the products tested, seemed to decrease re-epithelialization the most. Contrary to our results, Demling and DeSanti\textsuperscript{10} compared Acticoat with a standard Xeroform and 8-ply gauze dressing that was continually moistened with a 0.01\% neomycin and polymyxin solution, and found that silver increased re-epithelialization by more than 40\%, which was significant. They concluded that silver released into a moist environment significantly increased the rate of re-epithelialization compared with a standard antibiotic solution. Some degree of re-epithelialization was seen in the groups treated with Flamazine and PolyMem Silver. Aquacel Ag had the least effect on re-epithelialization, showing epithelial buds at the wound margins as well as an epithelial tongue that almost covered the entire wound bed. The controls incubated in 10\% FCS showed complete re-epithelialization and healing; whereas, the groups incubated in 2\% FCS showed no re-epithelialization after a 2-week incubation, as expected, as this corresponds to a chronic wound. None of the groups treated with products containing silver showed the same grade of re-epithelialization or healing as the controls incubated in 10\% FCS. All 7 products delayed re-epithelialization in this model.

**Conclusion**

A blackening of the wound bed is often transient binding of silver to wound debris that will be shed as the wound heals. However, as suggested in this study, silver might also be deposited deeper into the tissue. It can only be speculated as to what extent these deposits might be harmful to patients and their wounds. Silver deposited near blood vessels could cause damage, and silver might escape into the systemic circulation leading to silver protein complexes in different parts of the body (eg, skin, liver, kidney, bone marrow, or eyes).

When considering the effect of silver deposits in patients, the long-term effect of silver deposits in the dermal layer of the skin must be taken into account. The only variable between the positive control groups and the treatment groups was the application of products containing silver, which makes it possible to estimate the effect of silver on re-epithelialization in this model.

The question remains as to whether the amount of silver deposits in the tissue correlates with the antimicrobial properties of the silver. One can surmise that nanoparticles can penetrate further down and reach bacteria deeper into the tissue, thus exerting additional antibacterial activity. One can also surmise that the same particles are accumulated in the tissue, since they are too deep beneath the surface to be shed together with wound debris.

Before these results can be transferred to an *in-vivo* situation, one must consider systemic factors and other elements that are different *in vivo*. For example, the concentrations of applied silver on the wounds in a petri dish could be magnitudes greater than what would be applied to a metabolic unit, such as a chronic wound *in vivo*. Further study is needed to answer these questions.

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