



A comparative study of the cytotoxicity of silver-based dressings in monolayer cell, tissue explant, and animal models

Andrew Burd, MD, FRCS¹; Chi H Kwok, MPhil¹; Siu C Hung, MPhil¹; Hui S Chan, PhD²; Hua Gu, MPhil¹; Wai K Lam, PhD, FRSC(UK), FACB(US)²; Lin Huang, PhD¹

1. Division of Plastic and Reconstructive Surgery, Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong and

2. Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong

Reprint requests:

Professor Andrew Burd, MD, FRCSEd, FHKAM (Surgery), Department of Surgery, Prince of Wales Hospital, Hong Kong SAR, China. Tel: +852 2632 2639
Fax: +852 2632 4675;
Email: andrewburd@surgery.cuhk.edu.hk

Manuscript received: February 9, 2006
Accepted in final form: August 17, 2006

DOI:10.1111/j.1524-475X.2006.00190.x

ABSTRACT

Over the past decade, a variety of advanced silver-based dressings have been developed. There are considerable variations in the structure, composition, and silver content of these new preparations. In the present study, we examined five commercially available silver-based dressings (ActicoatTM, Aquacel[®] Ag, Contreet[®] Foam, PolyMem[®] Silver, Urgotul[®] SSD). We assessed their cytotoxicity in a monolayer cell culture, a tissue explant culture model, and a mouse excisional wound model. The results showed that ActicoatTM, Aquacel[®] Ag, and Contreet[®] Foam, when pretreated with specific solutes, were likely to produce the most significant cytotoxic effects on both cultured keratinocytes and fibroblasts, while PolyMem[®] Silver and Urgotul[®] SSD demonstrated the least cytotoxicity. The cytotoxicity correlated with the silver released from the dressings as measured by silver concentration in the culture medium. In the tissue explant culture model, in which the epidermal cell proliferation was evaluated, all silver dressings resulted in a significant delay of reepithelialization. In the mouse excisional wound model, ActicoatTM and Contreet[®] Foam indicated a strong inhibition of wound reepithelialization on the postwounding-day 7. These findings may, in part, explain the clinical observations of delayed wound healing or inhibition of wound epithelialization after the use of certain topical silver dressings. Caution should be exercised in using silver-based dressings in clean superficial wounds such as donor sites and superficial burns and also when cultured cells are being applied to wounds.

The use of silver as an antimicrobial agent has a long history.¹ Its recent resurgence follows from Moyer's use of silver nitrate solution in patients with burns.² Solutions gave way to cream formulations but it is now silver-containing dressings that provide the widest range of silver-based wound care products.^{3,4} Despite the ever increasing number of commercially available silver-based dressings, there is a distinct lack of comparative data on their clinical effectiveness.⁵ What is known is that silver can be effective against a wide range of microorganisms, including aerobic, anaerobic, Gram-negative and Gram-positive bacteria, yeast, fungi, and viruses. The antimicrobial effect of silver can be explained by various mechanisms: silver interferes with the respiratory chain in the cytochromes of micro-bacteria; additionally, silver ions also interfere with components of the microbial electron transport system, bind DNA, and inhibit DNA replication.^{6,7}

Dressings are designed to have more controlled and prolonged release of silver during the entire wear-time when compared with the cream formulations. This allows dressings to be changed less frequently, thereby reducing risk of nosocomial infection, cost of care, further tissue damage, and patient discomfort. Many factors affect the clinical performance of a dressing. The amount of silver content, the chemical, and physical forms of the silver, silver distribution, and even the affinity for moisture all participate in a dressing's capability to exert a significant antimicrobial effect. Marked differences exist in a variety

of silver dressing products. These products can be categorized as: (i) silver-delivery dressings such as ActicoatTM (Smith & Nephew, Hull, UK) and Urgotul[®] SSD (Laboratory URGD, Chenove, France), which have silver content coated (on the surface) or impregnated into the dressing material. They deliver silver to the wound site after direct contact; (ii) silver-containing dressings such as Aquacel[®] Ag (Cenvatec, Deeside, UK), Contreet[®] Foam (Coloplast, Humlebaek, Denmark), and PolyMem[®] Silver (Ferris Mfg. Corp., Burr Ridge, IL), which have high absorptive capacities and "lock up" the silver content until the dressing absorbs wound exudate or moisture. In

DMEM	Dulbecco's modified Eagle media
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGM	Fibroblast growth medium
ICP-MS	Inductively coupled plasma mass spectrophotometer
KGM	Keratinocyte growth medium
K-SFM	Keratinocyte serum-free medium
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
PWD	Postwounding day

Urgotul[®] SSD, the silver is in the form of silver sulfadiazine, which is released into the wound bed. This is metabolized to release silver and sulfadiazine moieties.⁸ Traditionally, the active silver agent has been thought to be ionic silver but in the nanocrystalline form, elemental silver is also thought to be active.⁹ Independent studies involving comparisons between different types of dressings suggested considerable variations in one or more aspects of dressings' performance.^{4,9–11} Our published microbiology data demonstrated that Acticoat[™] and Contreet[®] Foam have a broad spectrum of bactericidal activities against both Gram-positive and -negative bacteria, and Contreet[®] Foam was characterized by a very rapid bactericidal action.¹² However, we and others have also demonstrated in vitro cytotoxic effects of silver nitrate (AgNO₃), silver sulfadiazine, and Acticoat[™] on cultures of keratinocytes and fibroblasts.^{13–15}

In the present study, we are concerned with the cytotoxicity of a range of commercially available silver dressings to the viable cells in the wound bed. We have examined the biological effects of these dressings on: first, isolated skin cells—the monolayer culture of human keratinocytes and fibroblasts; second, the tissue explant culture model—pig mid-dermis culture for epidermal cell proliferation; and last, the mouse excisional wound model.

MATERIALS AND METHODS

Materials

Five silver-based dressings (Acticoat[™], Aquacel[®] Ag, Contreet[®] Foam, PolyMem[®] Silver, Urgotul[®] SSD) and

one control dressing without silver (Aquacel[®]) were obtained from commercial sources. The components of these dressings are summarized in Table 1.

Human keratinocyte and fibroblast cultures were developed from primary cultures of discarded surgical tissues or foreskins, according to Institution Ethical guidelines. 3T3/NIH fibroblasts were obtained from the American Type Culture Collection (ATCC; Rockville, MD). All the media and reagents used in cell cultures were purchased from GIBCO (Grand Island, NY) unless specified.

Six- to 8-month-old large white pigs and C57 BL/6J mice were sourced from the Laboratory Animal Services Centre of the Chinese University of Hong Kong (CUHK). All animal procedures were subject to the approval of the Animal Experimentation Ethics Committee of the CUHK, and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Keratinocyte culture

Keratinocytes were isolated from the skin tissues of discarded surgical tissues or foreskins as previously described.¹⁵ Briefly, surgical samples were collected in skin transport medium comprising Dulbecco's modified Eagle media (DMEM) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL Fungizone[®] (Invitrogen, Grand Island, NY), and 50 µg/mL gentamicin. The skin biopsy was transferred to the laboratory and trimmed by a surgical blade to remove all adipose tissue and cut into 2–3 mm wide strips before immersion in 2 mg/mL dispase II (Roche Diagnostics,

Table 1. Dressings

Dressing Name	Manufacturer	Basic dressing composition	Silver composition	Silver release
AQUACEL [®]	ConvaTec (Deeside, UK)	Hydrocolloid fiber (sodium carboxymethylcellulose)	None	—
AQUACEL [®] Ag	ConvaTec	Hydrocolloid fiber (sodium carboxymethylcellulose)	1.2% w/w ionic silver (silver nitrate)	Ag ⁺
Acticoat [™]	Smith & Nephew (Hull, UK)	An absorbent polyester inner core sandwiched between two outer layers of silver-coated polyethylene net	Metallic nanocrystalline silver	Ag ⁰
Contreet [®] Foam	Coloplast (Humlebaek, Denmark)	Polyurethane foam	Ionic silver (silver sodium hydrogen zirconium phosphate)	Ag ⁺
PolyMem [®] Silver	Ferris Mfg. Corp. (Burr Ridge, IL)	Polyurethane foam containing a safe nontoxic cleanser (F-68 surfactant), a moisturizer (glycerol) and an absorbing agent (superabsorbent starch copolymer)	Elemental nanocrystalline silver (124 µg/cm ²)	Ag ⁰
Urgotul [®] S.S.D	Laboratory URGO (Chenove, France)	Polyester gauze dressing impregnated with hydrocolloid particles dispersed in a Vaseline paste	Silver sulfadiazine	Ag ⁺

Basel, Switzerland) in DMEM for overnight digestion at 4 °C. The following day, the epidermis was mechanically separated from the dermis and collected into 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) solution. Basal keratinocyte cells were dissociated by vortex for 1 minute and neutralized with three volumes of DMEM with 10% FBS. The cells were then pelleted by spinning at 200×g for 5 minutes. Cells were resuspended in keratinocyte growth medium (KGM) consisting of one volume of Ham's F12, three volumes of DMEM, 10% FBS, 100 U/mL penicillin, 100 µL/mL streptomycin, 0.25 µg/µL amphotericin B, 100 fM cholera enterotoxin, 5 µg/mL transferrin, 180 µM adenine sulfate, 5 µg/mL insulin, 10 mg/mL epidermal growth factor, 0.4 µg/mL hydrocortisone (Sigma), and 20 fM liothyronine (Sigma). The cell suspension was seeded onto 4 µg/mL mitomycin C-treated-3T3/NIH feeder layer at a density of $3 \times 10^4/\text{cm}^2$. Keratinocytes were subcultured at 70–80% confluence after differential dissociation of the feeder layer with 0.02% EDTA for 10 minutes, followed by keratinocyte cell dispersion with 0.05% trypsin-0.02% EDTA treatment for 5 minutes. Cells were expanded one more passage on feeder cells and stored under liquid nitrogen in KGM with 5% dimethyl sulfoxide (DMSO) and 20% FBS. Cells at passages 3–5 were used in the following experimental assays.

Fibroblast culture

The dermis was separated from the skin tissues of discarded surgical tissues or foreskins by dispase digestion as previously described.¹⁵ The dermis was then finely minced, and resultant cell suspension, together with small pieces of tissues, were transferred to culture dishes and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µL/mL streptomycin, and 0.25 µg/µL amphotericin B (fibroblast growth medium, FGM). Medium was changed weekly. Upon reaching confluence, cells were passaged using 0.25% trypsin-EDTA. Cells at passages 4–10 were used in the following experimental assays.

Dressing absorbency test

The dressing absorbency test was performed according to the instructions from the “Test methods for primary wound dressings-Part 1: Aspects of absorbency” issued by the State Food and Drugs Administration, People's Republic of China, which is compatible with the European reference of Test methods for primary wound dressings—the EN 13726:2002 – section 3.2 free-swelling capacity. In brief, the testing dressing was cut into $1 \times 1 \text{ cm}^2$ size and was weighed (W_0). A solution A composed of 142 mmol Na ions and 2.5 mmol Ca ions was first prewarmed to 37 °C, and then the testing dressing as well as solution A were placed onto a 100 mm Petri dish and incubated at 37 °C for 30 minutes. The volume of solution A required was 40 times larger than the weight of the dressing. The dressing was weighed again after a 30-minute incubation (W_{30}). The absorbency of the dressing was expressed as volume of solution A absorbed per square centimeter dressing ($[W_{30}-W_0]/\text{area}$) and set as one of the parameters for dressing pretreatment as described below.

Pretreatment of dressing materials

All dressings were cut into $1 \times 1 \text{ cm}^2$ size under a sterile condition. Based on the dressing absorbency test result, PolyMem[®] Silver was found to have the highest absorbency of 0.8 mL/cm². The time for the foam dressings to be fully saturated with solution A was 10 minutes. Therefore, for the pretreatment, all the dressings were soaked with 0.8 mL of different solutes—deionized water, saline, and 100% FBS, respectively, for 10 minutes at 37 °C. The dressings, as well as individual pretreatment solutions, were then added to the culture for cytotoxicity assay.

Cytotoxicity testing of dressing materials on keratinocyte and fibroblast monolayer cultures

The cytotoxicity testing of six types of dressings on both keratinocyte and fibroblast monolayer cultures was performed in the same manner, except for using different culture medium and cell densities. Briefly, keratinocytes were seeded into six-well plates at a density of $1 \times 10^5/\text{well}$ and cultured in defined keratinocyte serum-free medium (K-SFM; GIBCO)-containing insulin, epidermal growth factor, and fibroblast growth factor. Fibroblasts were seeded at a density of $5 \times 10^4/\text{well}$ in FGM. Upon 3–4 days culture until 70–80% cell confluence, the $1 \times 1 \text{ cm}^2$ dressings were presoaked with 0.8 mL of deionized water, saline, or FBS, respectively, as mentioned above. Afterward, the dressings together with individual pretreatment solutions, and 2.2 mL of culture medium, were added to each of the culture wells. Addition of 0.8 mL of the plain solute without dressing being soaked was regarded as a positive control. The cells were then incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air for 2, 4, 6, and 24 hours. At each time point, dressings were removed and cell viability was determined by an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Experiments were performed in triplicate.

MTT assay

MTT is a yellow dye that is taken up by viable cells and converted to Formazan 8 (purple crystals). Change in color can be assessed spectrophotometrically to give an assessment of metabolic activity as a function of cytotoxicity.¹⁵ At each time point of the cytotoxicity testing, dressings from each well were removed and culture medium was aspirated. One milliliter MTT solution (0.5 mg/mL) in complete culture medium was added to each cell-containing well and then incubated in a humidified atmosphere for 2–4 hours in the dark. Afterward, 1 mL solubilization solution (10% SDS in 0.01 M HCl) was added and the plates were maintained at 37 °C in an incubator in a humidified atmosphere overnight. Afterward, 200 µL of the solubilization solution from each well was transferred to a 96-well enzyme-linked immunosorbent assay (ELISA) plate for spectrophotometric measurement using a microplate (ELISA) reader (Spectra Rainbow, TECAN, Grödig, Austria), with a test wavelength of 570 nm and a reference wavelength of 690 nm.

The results of the MTT assay were expressed as the relative cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD). The

statistical significance was assessed by Student's *t*-test using Prism 3.0 software from Graphpad, and $p < 0.05$ was considered to be significant.

Silver content of dressing materials

Samples of each dressing in $1 \times 1 \text{ cm}^2$ were digested by heating in a mixture of concentrated HNO_3 and HCl to break down the dressing matrix and to release and dissolve all of the silver present. The digest was then filtered and diluted with deionized water. Total silver in the aqueous samples was determined by inductively coupled plasma mass spectrometry (ICP-MS, 7,500c, Agilent Technologies Inc., Palo Alto, CA). Sample aerosol generated by a nebulizer was carried to an argon plasma of about 8,000 K for the production of silver ions. The sample ion was then introduced into a mass spectrometer for ion identification and quantification. For verification of the accuracy of the analyte, a standard reference material (SRM 1,577b, Bovine Liver, the National Institute of Standards and Technology, Gaithersburg, MD) was analyzed along with the samples. The sensitivity of the assay was 1.6 nmol/L. The interassay coefficients of variations were 3.7% at 38 nmol/L and 2.5% at 320 nmol/L. The total extractable silver content of each dressing was then determined and expressed in $\mu\text{g}/\text{cm}^2$.

Silver dissociation of dressing materials in different solution

To determine silver dissociation of each dressing in different solutions, dressing samples in $1 \times 1 \text{ cm}^2$ piece were presoaked with 0.8 mL of deionized water, saline or 100% FBS, respectively, for 10 minutes and forwarded to 2.2 mL culture medium, K-SFM, or FGM, respectively, for incubations at 37°C for 2, 4, 6, and 24 hours. The extraction solution was then centrifuged at 940g for 10 minutes at 4°C , and 200 μL of each supernatant was collected for ICP-MS assay as described above. The total amount of silver released over time was then determined and expressed in microgram per liter ($\mu\text{g}/\text{mL}$).

Pig mid-dermis explant culture

This model is based on a previously described technique.¹⁶ Briefly, mid-dermal sheets from the paravertebral areas of 6–8-month-old large white pigs were harvested under aseptic conditions using a Pagett's dermatome at a setting of 0.5 mm after removing a 0.5 mm thick split-thickness graft containing the epidermis. Dermal strips were cut into $1 \times 1 \text{ cm}^2$ segments and transferred onto sterile dressing gauze in Petri dishes to raise the explant to the air–liquid interface for culture. Orientation of the dermis was maintained. Explants were maintained in 5% CO_2 environment with 95% humidity in serum-free DMEM supplemented by 10 ng/mL hydrocortisone. The testing dressings of size $1 \times 1 \text{ cm}^2$ each were laid on the surface of the explants on day 0. The cultures were harvested at days 4 and 8, and subject to formalin fixation and rhodamine staining for visualization of the reepithelialized areas around the hair follicles. Resurfaced areas were photographed and their size was determined using computerized morphometric analysis (Metamorph 4.0). The ratio of area of reepithelialization

to the number of hair follicles in a certain area was determined to give an index of reepithelialization.

Mouse excisional wound model

This model is based on that described by Galiano et al.¹⁷ Four full-thickness excisional wounds extending through the panniculus carnosus were created on each C57 BL/6J mouse using a 6-mm diameter biopsy punch. A silicone splint was added onto each wound and sutured to reduce the contraction effect during wound closure. The wounds were either dressed with testing dressings or not dressed (control), followed by a cover of Tegaderm (a semi-occlusive dressing) to hold the dressings in place. At two time-points, post-wounding day 4 (PWD 4) and 7 (PWD 7), the whole-wound tissues were dissected and subjected to standard histological examination of the reepithelialization. The epithelial gap (EG) and wound gap (WG) of each wound were measured at microscopic level using image analysis software. The percentage of EG/WG (EGW) for each wound was then calculated as an indicator of wound-healing rate.

RESULTS

In vitro cytotoxicity of silver-based dressings on cultured human keratinocyte

As shown in Figure 1, the relative cell viability of keratinocytes after different dressing treatments varied significantly.

In control treatments in which three types of plain solute without dressing being soaked was individually added (see "Materials and Methods"), addition of serum (FBS) but not water and saline markedly suppressed the proliferation of keratinocyte. Addition of 0.8 mL of FBS in total volume of 3 mL culture medium led to a 30% reduction of cell viability at 24 hours when compared with that at 2 hours.

Aquacel[®], as a control dressing without silver, showed mild cytotoxicity on keratinocytes when delivered by water and saline. About 88 and 75% viable keratinocytes remained, respectively, after 24 hours dressing delivery by water and saline, respectively. When delivered in FBS, no additional reduction of cell viability was obviously observed compared with the control treatment.

Acticoat[™] exerted a severe cytotoxic effect on keratinocytes when delivered by water. Eighty percent of cells were killed immediately after the dressing was delivered with water for 2 hours. After 24 hours only $1.6 \pm 0.33\%$ viable cells remained. However, by using saline as a pretreatment solute, cell viability was significantly improved and remained around 80% during 24 hours. On the other hand, FBS pretreatment also improved cell viability but still led to 50% cell death after 24 hours.

Aquacel[®] Ag was cytotoxic to keratinocytes in all three pretreatment statuses. The relative cell viability at 24 hours was $53.1 \pm 5.04\%$, $9.0 \pm 5.80\%$, and $19.9 \pm 11.53\%$, respectively, when the dressing was delivered by water, saline, and FBS pretreatment, respectively.

Contreet[®] Foam was severely cytotoxic to keratinocytes when delivered by FBS. More than 95% cells were killed immediately after the dressing was delivered with FBS for 2 hours and the killing effect lasted till 24 hours. Using water and saline as the pretreatment solutes, this

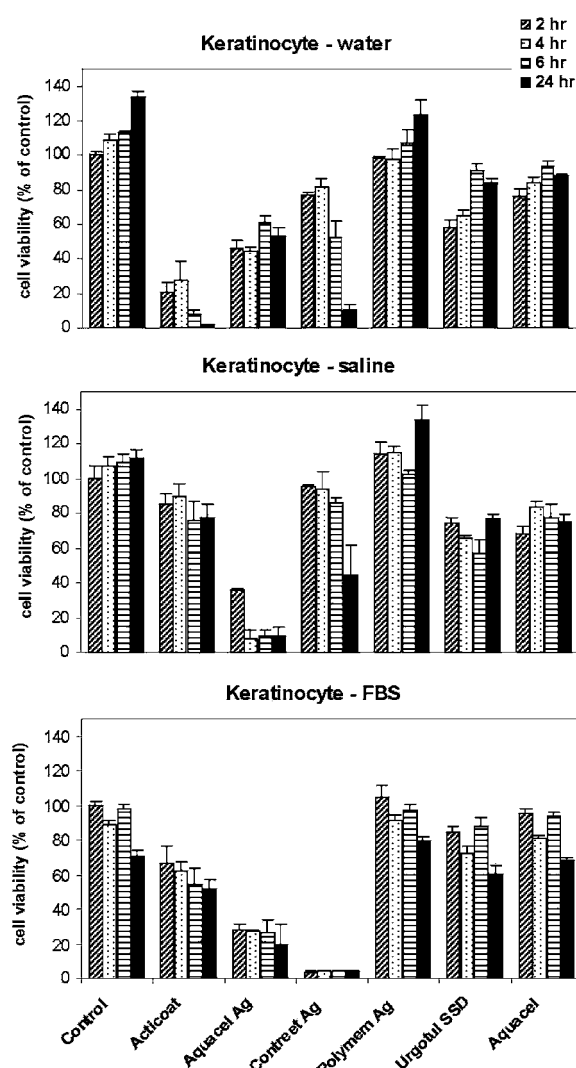


Figure 1. The relative cell viability of keratinocytes after various dressing treatments for 2, 4, 6, and 24 hours was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dressings were pretreated with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). Experiments were performed in triplicate. The results are expressed as percentage of the cell viability of individual experimental treatment to that of the control group at 2 hours (mean \pm SD).

dressing did not promote a significant cell death within a short period but led to 90 and 50% cell death, respectively, at 24 hours.

PolyMem[®] Silver was relatively safe for keratinocytes in all three pretreatment statuses. No obvious cytotoxic effect was observed in comparison with individual controls. In fact, the dressing was shown to enhance keratinocyte proliferation slightly when delivered by saline.

Urgotul[®]SSD was also relatively safe for keratinocytes. Its biological activity on the growth of keratinocyte was comparable with that of the nonsilver-containing dressing, Aquacel[®].

In brief, PolyMem[®] Silver was shown to be relatively safe for cultured keratinocytes, while Acticoat[™], Aquacel[®] Ag, and Contreet[®] Foam were significantly lethal when delivered by specific pretreatment solute.

In vitro cytotoxicity of silver-based dressings on cultured human fibroblast

As shown in Figure 2, the response of cultured fibroblasts to different dressing treatments also varied significantly.

In control treatments, the growth of fibroblasts was not significantly affected by the addition of the plain pretreatment solutes, water, and saline. While fibroblasts responded differently from keratinocytes to the addition of serum solute (FBS): no cell growth inhibition, rather a doubled increase of fibroblast proliferation, was observed within 24 hours.

Aquacel[®], as a nonsilver-containing dressing, hardly affected the cell growth of the fibroblast no matter which pretreatment solute was used. Within 24 hours of dressing delivery, fibroblasts proliferated similarly as they did in the control treatments.

Acticoat[™] exerted a significant cytotoxic effect on fibroblasts when delivered by water. Seventy percent of cells were killed immediately after the dressing was delivered with water for 2 hours, and after 24 hours, only $25.2 \pm 0.78\%$ viable cells remained. By using saline as a pretreatment solute, the cell viability of fibroblast was improved but still remained less than 50% at 24 hours. However, the cell viability was improved to more than 70% at 24 hours when the dressing was delivered with FBS.

Aquacel[®] Ag exerted severe cytotoxicity on fibroblasts no matter which pretreatment solute was used. The lethal effect was observed immediately after 2 hours of dressing delivery. Less than 25% fibroblasts could survive after being exposed to Aquacel[®] Ag for 24 hours.

Contreet[®] Foam was also severely cytotoxic to fibroblasts after 24-hour delivery. Among the three pretreatment solutes, a relatively slow onset of action was observed when the dressing was delivered by saline pretreatment, $63.7 \pm 1.87\%$ cells remained after 2 hours, while 60.1 ± 5.17 , 46.7 ± 8.36 , and $22.9 \pm 3.31\%$ viable cells remained after 4, 6, and 24 hours, respectively.

PolyMem[®] Silver was observed to show mild cytotoxicity to fibroblasts within a short period. At 6 hours of the dressing delivery, about 77.5 ± 0.00 , 63.1 ± 3.18 , and $82.8 \pm 1.21\%$ viable cells remained, respectively, in water-, saline-, or FBS-delivered treatments. After 24 hours, the cells seemed tolerant to all the treatments and started to proliferate again.

Urgotul[®]SSD affected fibroblast in a manner similar to PolyMem[®] Silver. Using saline as the pretreatment solute, around 30% cells were killed within 6 hours of the dressing delivery, while the cells were observed to proliferate again at 24 hours.

In brief, PolyMem[®] Silver and Urgotul[®]SSD were the least cytotoxic to cultured fibroblasts, while Acticoat[™], Aquacel[®] Ag, and Contreet[®] Foam showed significant killing or inhibitory effect on the cell growth of the fibroblast.

Silver content and silver dissociation of the dressings

The measured total silver content of the dressings is shown in Table 2, which indicates large differences between the

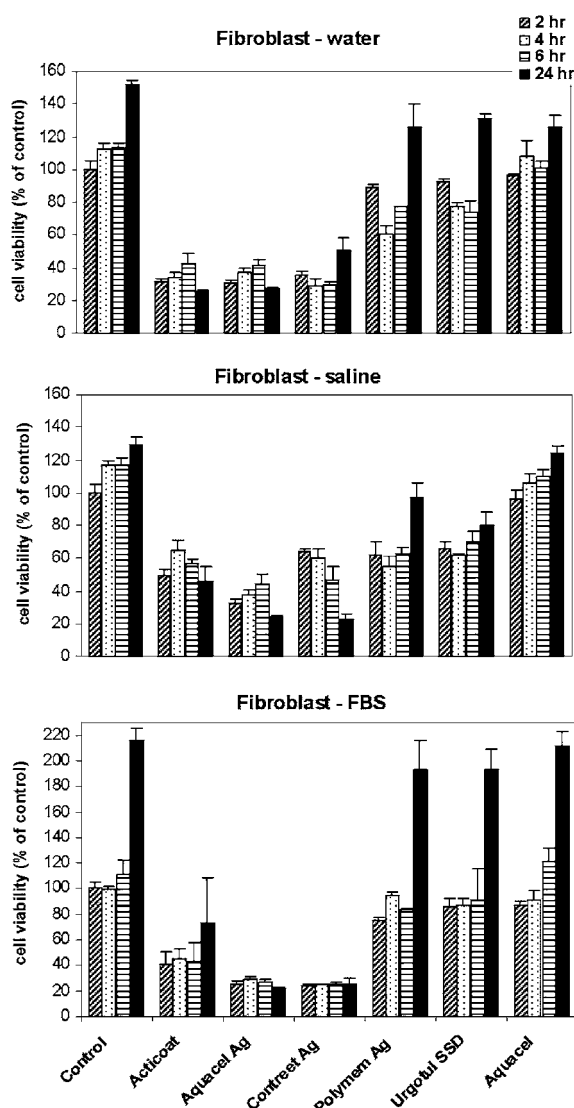


Figure 2. The relative cell viability of *Fibroblasts* after various dressing treatments for 2, 4, 6, and 24 hours was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dressings were pretreated with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). Experiments were performed in triplicate. The results are expressed as percentage of the cell viability of individual experimental treatment to that of the control group at 2 hours (mean \pm SD).

five products, ranging from 13 μg for Contreet[®] Foam to 934 μg for Acticoat[™] for a 1 cm^2 sample.

The amount of silver released into different culture medium including keratinocyte culture medium (K-SFM serum-free medium) and fibroblast growth medium (FGM—DMEM+10% FBS) after the dressing was pre-soaked with different solutes including water, saline, or FBS, also varied extensively over time. An increase in silver release was seen with the increase of the time. It is also noteworthy that, when the dressings were pre-soaked with

Table 2. Silver content of the dressings

	Ag content ($\mu\text{g}/\text{cm}^2$)
Acticoat [™]	934
Aquacel [®] Ag	21
Contreet [®] Foam	13
PolyMem [®] Silver	139
Urgotul [®] SSD	85

FBS, the amount of silver released into either keratinocyte culture medium or fibroblast growth medium was significantly improved, in particular, in the case of Acticoat[™], Aquacel[®] Ag, and Contreet[®] Foam (Figures 3 and 4).

Comparison of the silver release and silver content for different dressings revealed no correlation. Comparison of the in vitro cytotoxicity and silver dissociation in different pretreatment solutes and culture mediums revealed that, generally, there was an obvious correlation of these two factors for different dressings (Figures 3 and 4). For example, when compared with Acticoat[™], Aquacel[®] Ag, and Contreet[®] Foam, PolyMem[®] Silver, and Urgotul[®] SSD, two silver-containing dressings that showed the least cytotoxicity to both cultured keratinocytes and fibroblasts, released much less amount of silver into the culture medium over time. However, such a correlation was not always exactly consistent for highly cytotoxic dressings such as Acticoat[™], Aquacel[®] Ag, and Contreet[®] Foam. For example, in keratinocyte cultures, when pre-soaked with saline, Contreet[®] Foam showed the largest silver release at 24 hours (Figure 3D). Aquacel[®] Ag, however, not Contreet[®] Foam, was found to be the most cytotoxic under the same conditions (Figure 1).

In situ cytotoxic effect of silver-based dressings on epidermal reepithelialization

The cytotoxic effects of silver-based dressings on an epidermal cell proliferation model—pig mid-dermis explant culture—were also examined. As shown in Figure 5A, in the control cultures, the reepithelialization index (reepithelialization area [mm^2] per hair follicle) kept growing from days 0 to 8, while a delayed reepithelialization was observed in all explant cultures covered by silver-based dressings. The least number and area of rhodamine-stained reepithelialized surface was seen in the Acticoat[™]-dressed explant culture, whose reepithelialization index was 0.083 ± 0.005 and 0.089 ± 0.014 , respectively, at days 4 and 8. Histological examination (Figure 5B) also confirmed the significant reepithelialization in the control group, but not in samples treated with silver-based dressings.

In vivo cytotoxicity effects of silver-based dressings on wound reepithelialization

We used a mouse excisional wound model to study the effect of silver-based dressings on wound epidermal reepithelialization. As shown in Figure 6A, at the microscopic

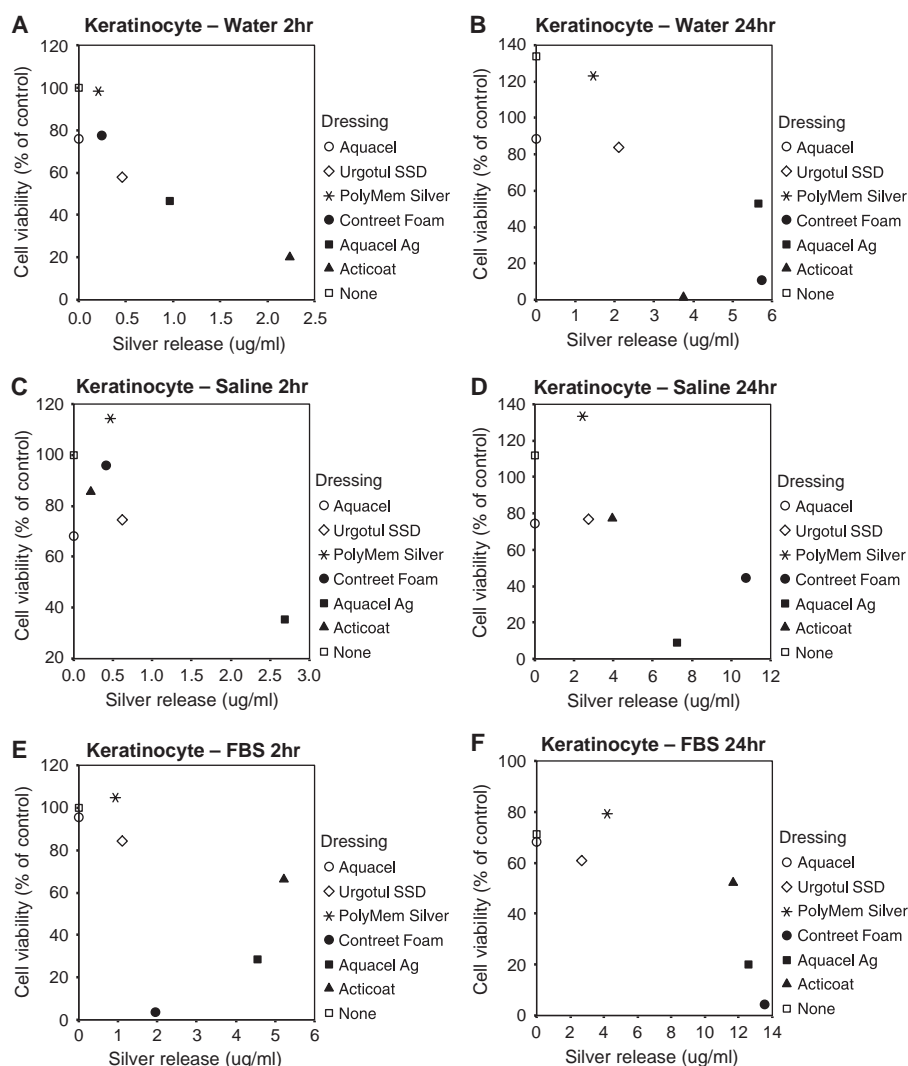


Figure 3. Comparison of the amount of silver released (ug/mL) into keratinocyte culture medium with the relative cell viability (%) after various dressing treatments for 2 and 24 hours. The dressings were presoaked with different solutes including water, saline, and fetal bovine serum (FBS; see details in “Materials and Methods”). The total amount of silver released into the culture medium over time was determined by an inductively coupled plasma mass spectrophotometer (ICP-MS) assay. A scatter diagram was plotted to analyze the relationship between the silver concentration and the cell viability.

level, the epithelial tongue (ET) comprised keratinocytes growing from the adjacent unwounded epidermis toward the center of the wound. The epithelial gap (EG) and wound gap (WG) of each wound were measured; the percentage of EG/WG (EGW) for each wound was then calculated as an indicator of wound-healing rate (the larger the EGW, the more the delay in reepithelialization). The data are summarized in Figure 6B. As shown, the inhibitory effect of silver-based dressings was not significant on PWD4. On PWD7, Acticoat™, and Contreet® Foam indicated a strong inhibition of reepithelialization, the EGW of which were 71.4 and 73.1%, respectively; in comparison, the EGW of the control group was 25.9% only on PWD7. Aquacel® Ag, and PolyMem® Silver also impaired wound reepithelialization on PWD.⁷

DISCUSSION

The results from this study serve to underline the complex differences between a small range of commercially availa-

ble dressings. At the outset, we sought to establish a “battery” of laboratory-based models which could be used to assess the potential clinical performance of the ever-rapidly proliferating range of silver-based dressings. Cell culture techniques are well established and highly reproducible and have been used by others and ourselves to assess silver toxicity.^{15,18} There are limitations, however, to using monolayer cultures eloquently described by Lansdown as “naked cells” in a review of silver in wound care.¹⁹ In our own clinical experience, we can acknowledge the value of hypochlorite solution in wound care which fell out of favor in the “Eusol” debate.¹⁵ Thus, in this evaluation, we sought to extend the models and add to the biological complexity by using a three-dimensional tissue explant model and an animal model, albeit murine, of dynamic epidermal cell proliferation. It is also relevant to observe that in our clinical practice, the major focus is on healing wounds where infection is more a feature to be prevented, than treated, and unimpeded cell proliferation is the biological means to the clinical end point of wound closure. Having said that, the antimicrobial effect of the silver

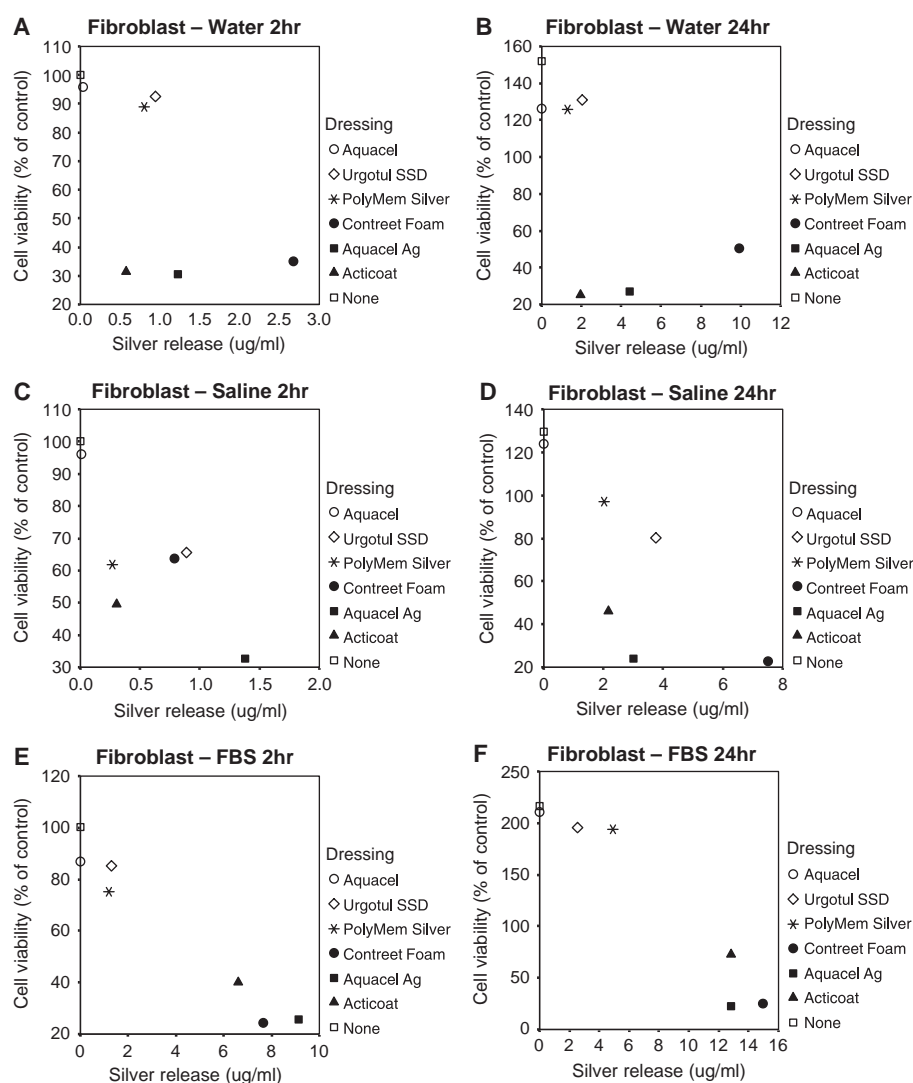


Figure 4. Comparison of the amount of silver released (ug/mL) into fibroblast culture medium with the relative cell viability (%) after various dressing treatments for 2 and 24 hours. The dressings were pre-soaked with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). The total amount of silver released into the culture medium over time was determined by an inductively coupled plasma mass spectrophotometer (ICP-MS) assay. A scatter diagram was plotted to analyze the relationship between the silver concentration and the cell viability.

based dressings remains a significant feature in our clinical application. It was anticipated that the ability of a dressing to exert a significant antimicrobial effect would be directly related to the silver content of the dressing and this was certainly demonstrated in one reported study.⁷ The situation appears to be far less clear in the cytotoxicity studies. Thus, for example the measured silver content of PolyMem[®] Silver was 139 $\mu\text{g}/\text{cm}^2$, which is tenfold higher than that of Contreet[®] Foam (13 $\mu\text{g}/\text{cm}^2$). Yet, PolyMem[®] Silver had a less apparent cytotoxic effect than Contreet[®] Foam. More critical determinants of the potential cytotoxicity of a dressing are the nature of the dressing, in particular, its affinity for moisture as well as the silver composition, that is to say, the distribution of the silver within or on the dressing and the chemical and physical form of the silver (metallic, bound, or ionic). In this study, there are two preparations of nanocrystalline silver: Acticoat[™] which delivers the silver from the surface, and, PolyMem[®] Silver, which is a foam-based dressing. We have demonstrated that PolyMem[®] Silver has the highest absorbance among the dressings tested in this study. It

also has less silver released into the carrier medium and thus it appears to be "locking up" the silver in the dressing. This is potentially a very good feature of a silver-based dressing where the bacterial "kill zone" is in the dressing rather than in the wound, thus avoiding the "collateral" damage to the healthy cells within the wound.

The test of absorbance that we have used is based on that described by the State Food and Drugs Administration, China, which in turn is based on / compatible with European reference tests. We have scaled down the amount of material used for cost reasons but in all other respects have found this does give a true and representative comparison with published data. It should be emphasized that we are not aiming to prove or disprove the claims of any commercially sponsored research but rather to look for fair, reasonable, and independent models to evaluate the comparative performance of present and future dressings. The correspondence regarding the validity of testing methodologies is insightful in this regard with the 20-second absorption time in Parson's Convatec-sponsored paper²⁰ being criticized in Anderson's response (on

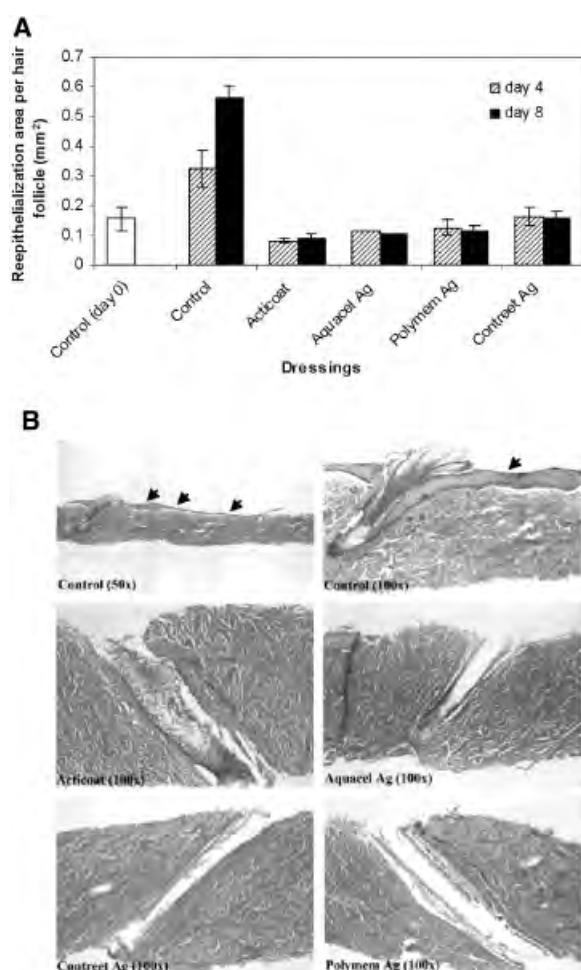


Figure 5. Silver dressings delay epidermal reepithelialization in pig mid-dermis explant culture. (A) The reepithelialization index (reepithelialization area [mm²] per hair follicle) on days 4 and 8 postdressing delivery was determined ($n=3$). (B) Examples of histological examination (H&E staining) of cultured tissue explants on day 8 for different experimental treatment groups. The arrows represent reepithelialization areas.

behalf of Coloplast).²¹ It is also interesting that in the ensuing correspondence, the validity of methodology to determine silver release has also been challenged.

The decision to use a "pretreatment" solution was based on the clinical application of the dressing. According to the manufacturer's instruction, ActicoatTM needs to be moistened with deionized water before use. Saline is expressly not to be used. Other dressings may be applied dry or moistened. The interaction with the wound will depend upon the fluid environment. For silver to be in a biologically active form, it must be soluble either as Ag⁺ or Ag⁰ clusters. Ag⁰ is the metallic or unchanged form of silver found in the nanocrystalline formulations.⁹

What is perhaps surprising is the effect of the pretreatment solution on the cytotoxicity of the various dressings. As an example, ActicoatTM was found to have a significant cytotoxic effect on both keratinocytes and fibroblasts

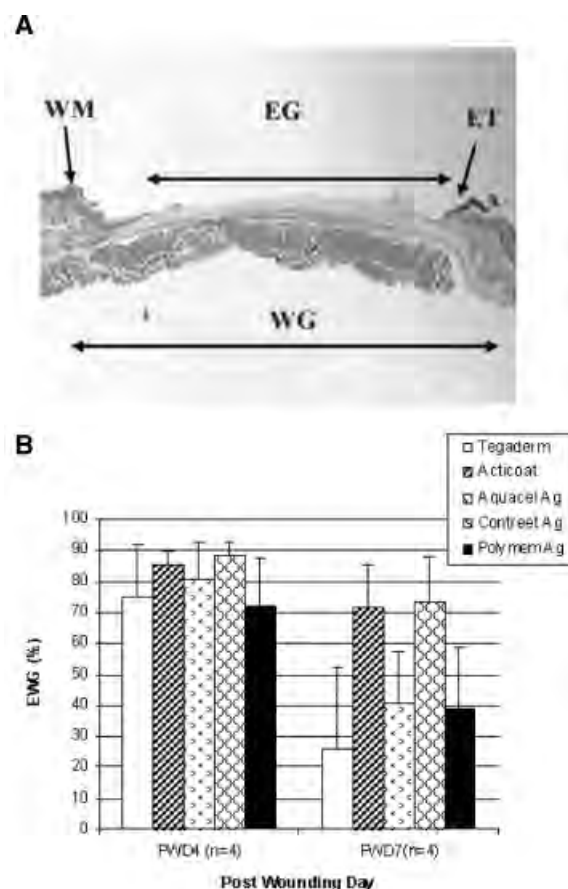


Figure 6. Silver dressings inhibit wound reepithelialization in a mouse excisional wound model. (A) An example of the histological examination (H&E staining) of wound section on postwounding day 4. WM, wound margin; ET, epithelial tongue; EG, epithelial gap; WG, wound gap. (B) Quantitative analysis of wound reepithelialization on postwounding days 4 and 7 for different experimental treatment groups. Data are expressed as the percentage of EG/WG (i.e., EWG %).

when pretreated with water. Pretreatment with saline significantly reduced the cytotoxicity. The release of silver from ActicoatTM pretreated in saline was significantly reduced when compared with ActicoatTM pretreated with water. The variation in the measured amount of silver released is again surprising. It must be appreciated, however, that the model system is far more complex than that used when manufacturers measure silver release. Thus, the data provided from a Coloplast-sponsored study of in vitro release profiles used a diffusion cell with a continuous flow of 1.4 mL/hour of release media consisting of an isotonic solution with equal amounts of sodium and serum.²² In our system, the dressings were pretreated and then allowed to equilibrate in two different types of media in which the two cell types were grown. These media are both complex protein-containing solutions.

It was found in the present study that under all test conditions, Aquacel[®] Ag and Contreet[®] Foam released a comparable or even greater amount of silver ions than

Acticoat™ at 24-hours postdressing delivery. This, to some extent, was found to lead to a greater cytotoxic effect than Acticoat™. It is also noteworthy that when the dressings were presoaked with FBS, the amount of silver released into both keratinocyte culture medium (K-SFM) or fibroblast growth medium was significantly increased, in particular, in the case of Acticoat™, Aquacel® Ag, and Contreet® Foam. Serum is an extremely complex mixture of plasma proteins, growth factors, hormones, etc. The mechanism involved in serum-promoted silver release is yet unknown, but clearly and generally, the presence of sodium and chloride ions has an effect on silver dissociation.

The pig mid-dermis model is an explant culture model. It provides a three-dimensional system that can also be analyzed in four dimensions (time being the fourth dimension). We have previously used this model to assess the stimulation of cell proliferation and migration by the exogenous application of topical agents.¹⁶ The use of a highly reproducible biological model was appealing and could also provide statistically appropriate comparative data when looking at the area of reepithelialization. In this study we have also compared vertical sections of the explants and selected those that showed clear sections of the hair follicle shaft. We have not elaborated a scoring system for this aspect and so the comparison becomes descriptive rather than statistical. Nevertheless, it is interesting to note that in all four dressings applied, the reepithelialization was not noted. As with other aspects of this current study, there is the potential to explore the biological mechanisms in more detail for example using immunohistochemical staining of matrix protein. This is, however, beyond the scope of this paper. The mouse wound-healing model again focuses on reepithelialization. The typical wound would quickly heal by contraction so the silicone splint keeps it open to allow closure by reepithelialization to be measured. Both models demonstrated delayed or inhibited reepithelialization by silver-based dressings. Taken together, our findings may explain the clinical observation of delayed wound healing or inhibition of wound epithelialization after the use of topical antimicrobial dressings. We suggest that silver-based dressings should be used with caution in situations where rapidly proliferating cells may be harmed as in donor sites, superficial burns, and application of cultured cells. It must be observed that previous studies that demonstrate the silver enhances acute wound healing were performed on incisional wounds where keratinocyte proliferation is not a major feature.²³

Silver dressings are used in a wide range of wound-healing situations. In our clinical practice, we are more involved in the acute wounds and the emphasis is more on the prevention of infection and promotion of healing. It was the study of the clinical data pertaining to Acticoat™ and reepithelialization that caused our initial questioning of the validity of such studies. On the one hand, Innes et al. demonstrated that nanocrystalline silver impedes reepithelialization in donor sites,²⁴ while Demling and DeSanti were demonstrating that it increases the rate of reepithelialization in meshed skin grafts.²⁵ Is one or another of these studies flawed or is there an explanation for both to be correct?

We also have to recognize the clinical anomalies of positive cultures being grown from wounds that have been dressed with silver-based products. This cannot simply be attributed to silver resistance, which is in fact quite rare.²⁶

Rather, it may well be that the bacteria in vivo behave differently from the bacteria in vitro such that silver is not such an effective killer in the wound as it is in the laboratory.

The requirements of a dressing in a chronic wound-healing situation are different where the control of the wound bioburden is more important.²⁷ Of course, this was one of the strengths of the hypochlorite solutions that ran into disrepute because of their laboratory-based toxicity.¹⁵ It is a concern when undertaking a study of commercially available materials that results will be quoted out of context. It is certainly not our intention to endorse or criticize any specific dressings. Rather, it has been our intention to explore the variability of the performance in the model systems described and thereby gain a greater understanding of the potential biological interactions of silver and wounds. It is important to keep in vitro derived data in perspective but this applies not only to the cell cytotoxicity of silver products but also their ability to effectively deliver silver and kill bacteria in vivo. Ultimately, the best evidence to support clinical effectiveness will come from randomized prospective blinded studies. Such comparative studies are awaited.

ACKNOWLEDGMENT

This work was supported by the Innovation and Technology Fund, the Government of the Hong Kong SAR (to Prof. A. Burd, ITS/086/03).

REFERENCES

1. Klasen HJ. Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns* 2001; 26: 117–30.
2. Klasen HJ. A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver. *Burns* 2001; 26: 131–8.
3. Thomas S, McCubbin P. A comparison of the antimicrobial effects of four silver-containing dressings on three organisms. *J Wound Care* 2003; 12: 101–7.
4. Thomas S, McCubbin P. An in vitro analysis of the antimicrobial properties of 10 silver-containing dressings. *J Wound Care* 2003; 12: 305–8.
5. Bergin SM, Wraight P. Silver based wound dressings and topical agents for treating diabetic foot ulcers. *Cochrane Database Syst Rev* 2006; 25: CD005082.
6. Lok CN, Ho CM, Chen R, He QY, Yu WY, Sun H, Tam PK, Chiu JF, Che CM. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *J Proteome Res* 2006; 5: 916–24.
7. Lansdown ABG. Silver I: its antibacterial properties and mechanism of action. *J Wound Care* 2002; 11: 125–30.
8. Lansdown ABG. A guide to the properties and uses of silver dressings in wound care. *Professional Nurse* 2005; 20: 41–3.
9. Dunn K, Edwards-Jones V. The role of Acticoat with nanocrystalline silver in the management of burns. *Burns* 2004; 30 Suppl. 1): S1–9. Review.
10. Walker M, Hobot JA, Newman GR, Bowler PG. Scanning electron microscopic examination of bacterial immobilisation in a carboxymethyl cellulose (AQUACEL) and alginate dressings. *Biomaterials* 2003; 24: 883–90.

11. Bowler PG, Jones SA, Walker M, Parsons D. Microbicidal properties of a silver-containing hydrofiber dressing against a variety of burn wound pathogens. *J Burn Care Rehab* 2004; 25: 192–6.
12. Ip M, Lui SL, Poon VK, Lung I, Burd A. Antimicrobial activities of silver dressings: an in vitro comparison. *J Med Microbiol* 2006; 55 (Part 1): 59–63.
13. McCauley RL, Linares HA, Pelligrini V, Herndon DN, Robson MC, Heggers JP. In vitro toxicity of topical antimicrobial agents to human fibroblasts. *J Surg Res* 1989; 46: 267–74.
14. McCauley RL, Li YY, Poole B, Evans MJ, Robson MC, Heggers JP, Herndon DN. Differential inhibition of human basal keratinocyte growth to silver sulfadiazine and mafenide acetate. *J Surg Res* 1992; 52: 276–85.
15. Poon VK, Burd A. In vitro cytotoxicity of silver: implication for clinical wound care. *Burns* 2004; 30: 140–7.
16. Burd DA, Greco RM, Regauer S, Longaker MT, Siebert JW, Garg HG. Hyaluronan and wound healing: a new perspective. *Br J Plast Surg* 1991; 44: 579–84.
17. Galiano RD, Michaels J, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen* 2004; 12: 485–92.
18. Hidalgo E, Bartolome R, Barroso C, Moreno A, Dominguez C. Silver nitrate: antimicrobial activity related to cytotoxicity in cultured human fibroblasts. *Skin Pharmacol Appl Skin Physiol* 1998; 11: 140–51.
19. Lansdown ABG. A review of the use of silver in wound care: facts and fallacies. *Br J Nurs* 2004; 13 (Suppl. 6): S6–19.
20. Parsons D, Bowler PG, Myles V, Jones S. Silver antimicrobial dressings in wound management: a comparison of antibacterial, physical, and chemical characteristics. *Wound* 2005; 17: 222–32.
21. Anderson MB. Letter to Editor. *Wound* 2006 March.
22. Dolmer M, Larsen K, Jensen K. In vitro silver release profiles for various antimicrobial dressings. Paper presented at the 2nd WUWHS Meeting, Paris, July 2004.
23. Lansdown AB, Sampson B, Laupattarakasem P, Vuttivirojana A. Silver aids healing in the sterile skin wound: experimental studies in the laboratory rat. *Br J Dermatol* 1997; 137: 728–35.
24. Innes ME, Umraw N, Fish JS, Gomez M, Cartotto RC. The use of silver coated dressings on donor site wounds: a prospective, controlled matched pair study. *Burns* 2001; 27: 621–7.
25. Demling RH, DeSanti L. The rate of re-epithelialization across meshed skin grafts is increased with exposure to silver. *Burns* 2002; 28: 264–6.
26. Ip M, Lui SL, Chan SS, Lung I, Burd A. The prevalence of resistance to silver in a burns unit. *J Hosp Infect* 2006; 63: 342–4.
27. Lansdown ABG, Williams A, Chandler S, Benfield S. Silver absorption and antibacterial efficacy of silver dressings. *J Wound Care* 2005; 14: 155–60.