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Reduced Cellular Toxicity of a New Silver-Containing Antimicrobial Dressing and Clinical Performance in Non-Healing Wounds

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Key Words
Wounds · Antimicrobial activity · Cell toxicity · Silver dressing

Abstract
Bacterial colonisation of wounds may delay wound healing. Modern silver-containing dressings are antimicrobial, yet cellular toxicity is a serious side-effect. We provide data for a newly formulated silver-containing ointment dressing, Atrauman Ag, for antimicrobial activity and cytotoxicity. Atrauman Ag effectively killed a panel of commensal skin as well as pathogenic bacterial strains while cytotoxicity for HaCaT keratinocytes was only around 10%. With these favourable in vitro tests, Atrauman Ag was analysed in 86 patients with traumatic and non-healing wounds of different aetiologies. The wound state was evaluated for 3 subsequent dressing changes. The slough score was reduced from 59.2 to 35.8%, granulation tissue increased from 27 to 40% and epithelialisation went up from 12.1 to 24%. We conclude that Atrauman Ag has a superior profile of antimicrobial activity over cellular toxicity and the low silver ion release rate may prevent interference with wound-healing mechanisms.

Introduction

Acute wounds are exposed to numerous micro-organisms resident on the skin or from the environment. If the repair process is compromised and non-healing wounds arise, these can become heavily contaminated. Colonisation is usually polymicrobial, containing numerous different micro-organisms some of which are potentially pathogenic. Colonisation alone in the absence of clinical signs of infection does not seem to impair wound healing, but with clinical symptoms, the exudative phase is prolonged and wound healing is further delayed. Patients suffer increased pain, treatment costs rise and it may sometimes take months or even years for wounds to close [for a review, see 1].

Irrespective of the causative micro-organisms, there is widespread debate when and how to initiate treatment of infected wounds. Furthermore, treatment of infected wounds with antibiotic-resistant bacteria, e.g. methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, pose a major problem in wound care [2].

The development of silver-containing wound dressings has markedly improved the local management of critically colonised and infected wounds [3]. Silver ions are released from the dressing into the wound fluid or

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exudate and act against a wide spectrum of pathogens such as aerobic, anaerobic, gram-negative and gram-positive bacteria, yeasts and viruses [4]. These types of dressings are particularly advantageous in cases where systemic antibiotic treatment is not needed, but where local antimicrobial activity is desired. In addition, the long-lasting antimicrobial action and the significantly lower propensity to induce bacterial resistance is a further advantage.

Unfortunately, released silver ions are cytotoxic to human cells [5], and there is an inherent problem balancing antimicrobial activity against cytotoxicity [6]. Technically, this issue can be addressed by controlling silver release by varying the amount of available silver in the dressing, the surface area of the silver particles and the chemical composition of the silver preparation. In this study, we provide data on antimicrobial effects and HaCaT cytotoxicity of a new silver-containing dressing, Atrauman Ag, in comparison with two widely used silver-containing dressings, Acticoat and Actisorb silver 220. Furthermore, we analysed the performance of the new Atrauman Ag dressing in non-healing and traumatic wounds of different aetiologies.

Material and Methods

Dressing Types

Atrauman Ag is a new, silver-coated polyamide dressing impregnated with a mixture of adipic acid triglyceride esters and triglycerides. The silver is present on the surface of the polyamide fibres in a metallic form. Acticoat (Smith & Nephew, Lohfelden, Germany) and Actisorb silver 220 (Johnson & Johnson Wound Management, Norderstedt, Germany) were included as reference products.

Antimicrobial Activity of Atrauman Ag

The antimicrobial activity of Atrauman Ag was tested against a variety of bacteria (table 1). The test series were carried out according to standard methods of the American Society for Testing Materials [ASTM 2180; 'Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) in Polymeric or Hydrophilic Materials']. In brief, melted agar was inoculated with the different bacterial strains and dispensed onto the dressings to be analysed. The thin agar layer had close contact with the dressing and was incubated for 0, 2, 4, 6 and 24 h. The surviving bacteria were retrieved from the agar and counted by limited dilution plating. Antimicrobial activity was calculated from the number of bacteria at the beginning of the experiment and the number of viable bacteria after incubation with the dressings.

To test for the antimicrobial capacity over 9 days, fresh *S.-aureus*-containing agar was inoculated onto Atrauman Ag for 24-hour periods. Again, viable bacteria were quantified by the limited dilution method and colony counts. Experiments were repeated twice showing deviations of less than 5% between the measurements.

Table 1. Bacteria included in the study

Strain	Source
Staphylococcus aureus	ATCC 6538P
Staphylococcus aureus (methicillin-resistant)	ATCC 6538
Staphylococcus epidermidis	ATCC 12228
Klebsiella pneumoniae	ATCC 4352
Pseudomonas aeruginosa	ATCC 27853
Escherichia coli	ATCC 25922
Bacillus subtilis	ATCC 6633

Table 2. Patient demographics and underlying disease

Demographic variables	
Mean age ± SD, years	73 ± 15
Female, %	64
Male, %	36
Treatment duration, days	9 ± 3.7
Interval between dressing changes, days	3.1 ± 1.2
Wound characteristics	
Duration of the wound, years	1.1 (median 2 months)
Wound size, cm	4 ± 3.2 × 3.3 ± 3.4
Underlying disease, %	
Venous leg ulcers	31
Mixed leg ulcer	25.3
Acute wounds (traumatic)	18.4
Burns	8.0
Decubitus	5.7
Other	11.6
Total	100

Cytotoxicity Assays

Cytotoxicity of the different silver-containing dressings was tested by incubating serial dilutions of dressing-conditioned medium with the human keratinocyte cell line HaCaT [7]. While HaCaT keratinocytes differ in several aspects from primary keratinocytes, this cell line offers advantages in standardised tests by elimination of the interindividual variation of isolated cells. For each of the dressings, an eluate was produced by incubating 1 ml cell culture medium per 6 cm^2 area at 37°C for 24 h with constant agitation, 300 revolutions/min. For Atrauman Ag, the ointment-free dressing base was used to exclude any confounding effects from the ointment itself. The resulting eluate was sterile-filtrated and initially diluted 1:4.

The cytotoxicity testing was performed following the ISO 10993 procedure. In short, HaCaT keratinocytes were seeded into 96-well plates at 1×10^4/well. After 24 h, the cell culture medium was aspirated and replaced by dilutions of the dressing-incubated eluate (or plain medium for controls, or 5-fluorouracil at 10^{-4} *M* as positive control in the cytotoxicity assays). After 72 h, viability was tested by the MTT test [8] and the metabolically converted formazan was quantified spectrophotometrically at OD_{570}.

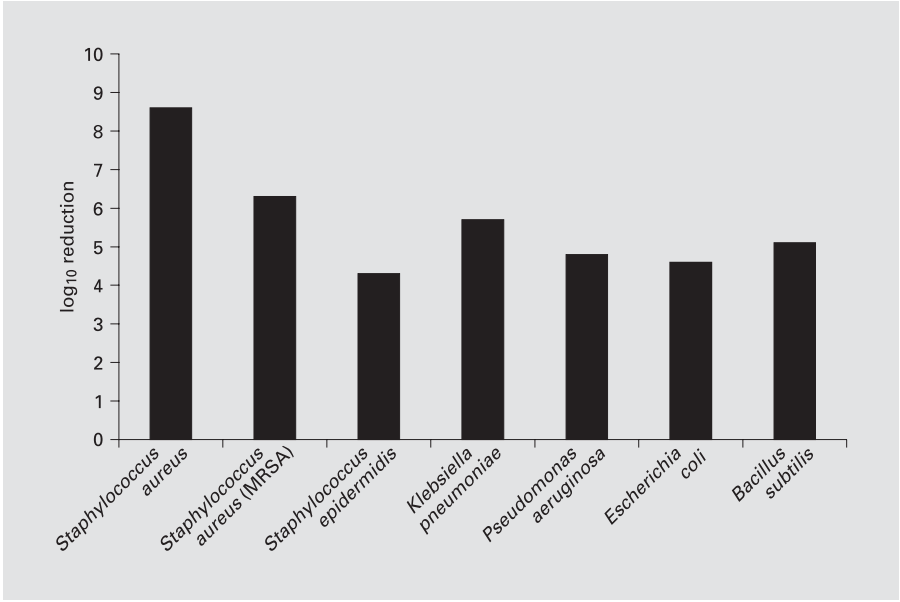

Fig. 1. Antimicrobial activity of Atrauman Ag. Different bacterial strains were inoculated onto Atrauman Ag in melted agar and incubated for 24 h. The viable bacteria were released and counted by limited dilution. The reduction was calculated from untreated controls and expressed after logarithmic transformation. All strains tested showed at least a reduction by 4 $\log_{10}$ steps. MRSA = Methicillin-resistant *S. aureus.*

Clinical Observational Study

The clinical performance of Atrauman Ag was assessed in an observational, descriptive out-patient study following the guidelines of the Ethics Committee of the Landesärztekammer Baden-Württemberg. 16 centres recruited 86 patients with non-healing wounds with a variety of different underlying diseases (table 2). Wound swabs or tissue biopsies for quantitative analysis were not requested at the entry of the study as collection and logistic processes are often prone to give misleading results in an office-based setting. The performance of Atrauman Ag was measured with a standard questionnaire describing the wound size, appearance and handling of the product. At the beginning and after 3 dressing changes, the wound state was assessed and quantified by the percentage of slough/eschar, exudation, granulation and epithelialisation. In addition, any patient who reported pain at dressing changes was recorded. Interobserver variances in assessment were minimised by definition of the items recorded and the number of participating centres.

Results

Antimicrobial Efficacy of a New Silver-Containing Polyamide Mesh

Atrauman Ag was effective against a methicillin-resistant *S. aureus* strain as well as numerous other gram-positive and gram-negative bacteria, in fact, all bacteria tested (fig. 1). With an initial count of 10^6 bacteria/ml, complete killing was achieved for *Klebsiella pneumoniae* after contact with the dressing for 2 h and for *S. aureus* after 4 h (data not shown). At 10^7 bacteria/ml, Atrauman Ag killed all bacteria within 24 h (fig. 2a). The duration of antimicrobial efficacy was demonstrated in long-term experiments. Atrauman Ag effectively eradicated both *S. aureus* and *K. pneumoniae* – despite repeated inoculation with bacteria onto the same dressing for 9 days (fig. 2b).

Cellular Cytotoxicity of Three Silver-Containing Dressings

As even low amounts of free silver ions possess high cellular cytotoxic potential, we next analysed the viability of HaCaT cells, an immortalised human keratinocyte cell line. Out of the 3 silver-containing dressings Atrauman Ag had the lowest cytotoxic effect with a viability of 90% (fig. 3). Viability of keratinocytes with the second dressing was slightly lower with values of 80% and one of the dressings, Acticoat, showed high cytotoxicity with only few cells surviving. To correlate the cytotoxicity data with the concentration of released silver ions we determined the silver content in the eluate. Here, the highly cytotoxic Acticoat released high concentrations of 71.4 ppm while for Actisorb silver 220 we measured values of 0.38 ppm and for Atrauman Ag 2.3 ppm.

Clinical Performance

86 patients with traumatic and non-healing wounds receiving conservative treatment before were followed for 3 consecutive dressing changes. The patient demographics and underlying diseases are listed in table 2. Most frequently, Atrauman Ag was used for treatment of venous

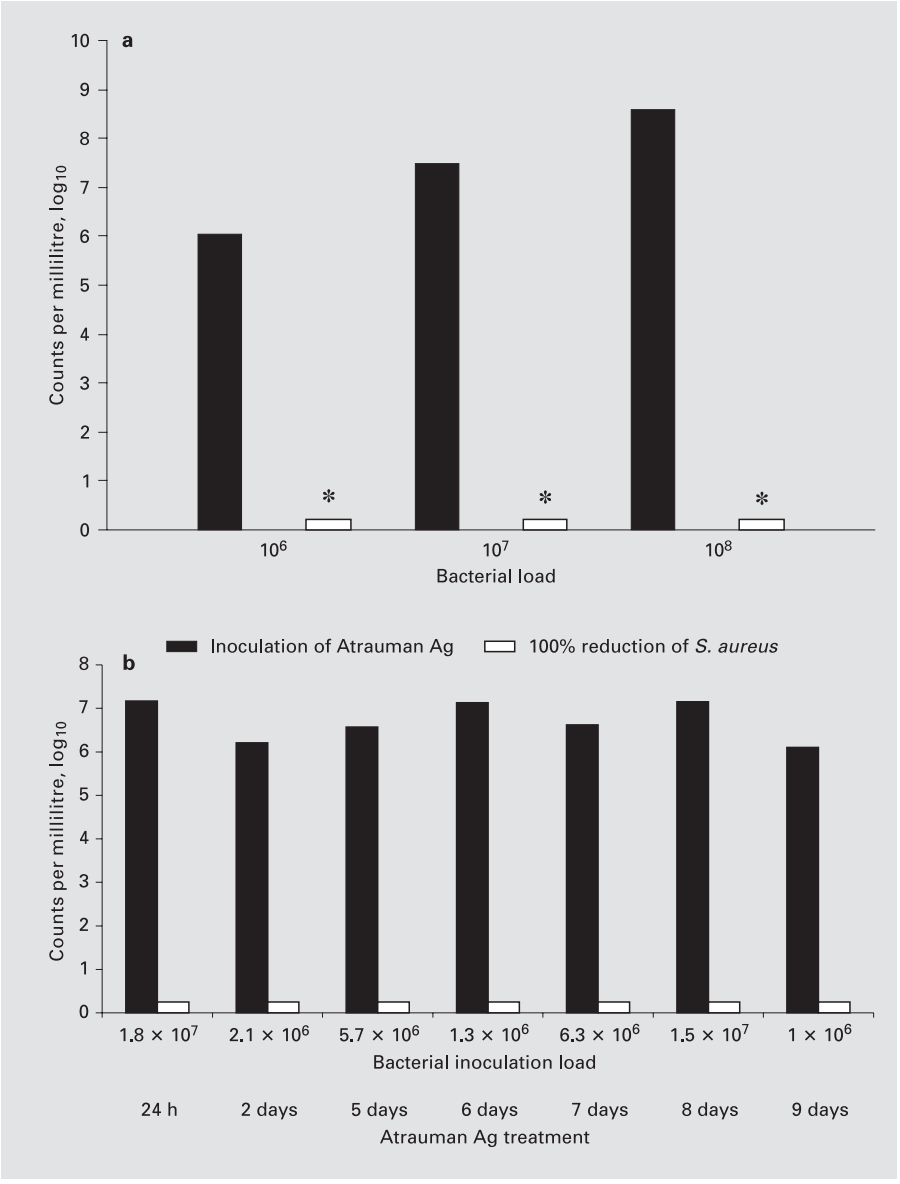

Fig. 2. a Antimicrobial activity of Atrauman Ag at different bacterial loads. *S. aureus* was inoculated at a density of 10^5, 10^6 and 10^7 bacteria/ml onto Atrauman Ag. After 24 h, viable bacteria were released and counted by limited dilution. Under these conditions, treatment with Atrauman Ag resulted in an almost complete reduction of viable bacteria. Asterisks indicate values below the detection limit. **b** Antimicrobial activity of Atrauman Ag in long-term experiments. *S. aureus* was inoculated onto Atrauman Ag in 24-hour steps for 9 consecutive days. After each 24-hour period, viable bacteria were released and counted by limited dilution before fresh bacteria were inoculated again. Under these conditions, treatment with Atrauman Ag resulted in an almost complete reduction of viable bacteria throughout the 9-day period. The variations of the controls are due to the slightly different growth rates of the individual inoculations. Closed columns represent the number of inoculated *S. aureus*. Open columns correspond to bacterial counts after incubation with Atrauman Ag.

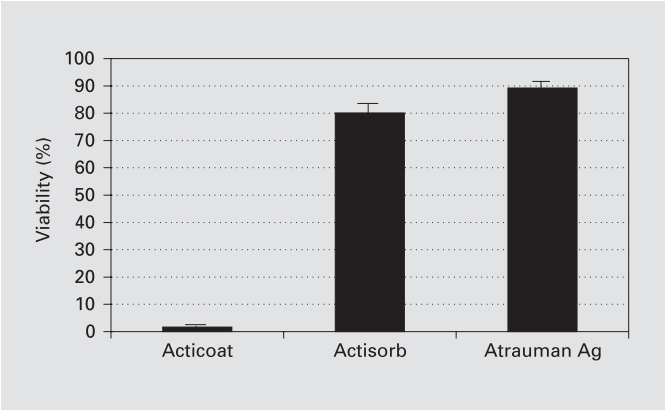

Fig. 3. Vitality of HaCaT keratinocytes after treatment with conditioned media from 3 different silver-containing dressings. Cytotoxicity was tested by incubating dilutions of dressing-conditioned medium with HaCaT keratinocytes in 96-well plates. After incubation, viability was tested by the MTT test and quantification of the converted and solubilised formazan at OD_{570}. Atrauman Ag was least cytotoxic followed by Actisorb 220 and Acticoat resulting in a viability of only 2%.

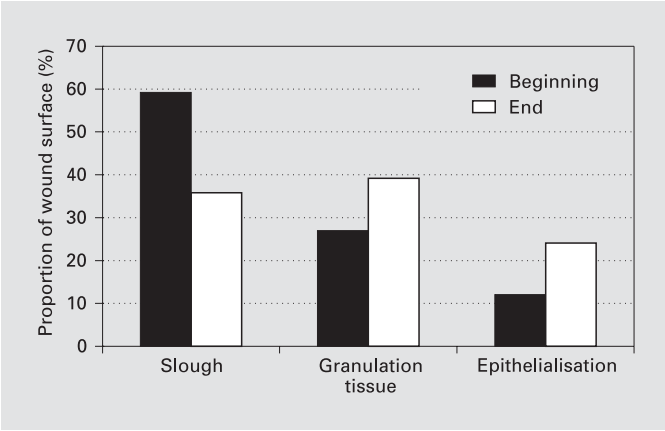

Fig. 4. Evaluation of wound appearance after treatment with Atrauman Ag for 3 consecutive dressing changes. Wounds of different aetiologies were treated for 3 consecutive dressing changes with Atrauman Ag. Subjective evaluation was recorded at each dressing change, and the results are shown as difference comparing the start and end of the observation period.

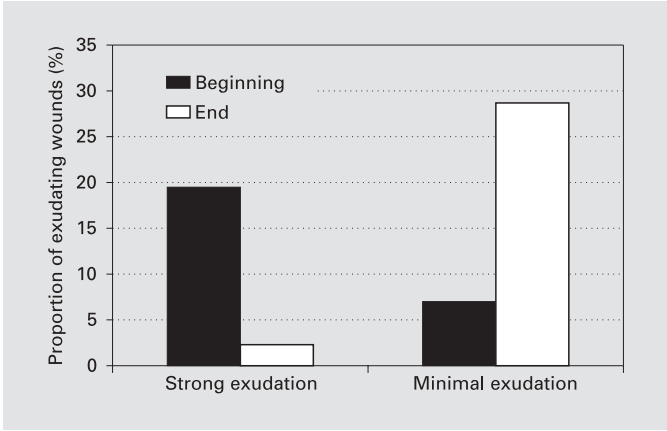

Fig. 5. Assessment of wound exudation over a period of 3 dressing changes of Atrauman Ag. Wounds were evaluated for exudation over the period of 3 consecutive dressing changes with Atrauman Ag. There was a reduction of wound exudation over the observational period suggesting – with data from figure 4 – an improvement of wound healing from the beginning to the end of the observation period.

leg ulcers (31%), and 25% of the wounds were mixed ulcers due to venous hypertension and reduced arterial perfusion.

At the beginning of treatment with Atrauman Ag, 59.2% of the wound surface was covered with slough, granulation tissue was apparent in 27% and 12.1% showed epithelialisation. After 3 dressing changes, the relative proportion had changed to 35.8% for slough, 40% for granulation tissue and 24% for epithelialisation (fig. 4). In addition, exudation decreased during treatment with Atrauman Ag. At the beginning, 19.5% were characterised as strongly exuding reduced to 2.3% at the end of the observation period and the percentage of minimally exuding wounds increased from 7 to 28.7% (fig. 5). The surface measurements were almost unchanged during the observation period.

The patient-reported pain sensation during dressing changes decreased from 78 to 45% in the course of the 3 dressing changes. 83% of the physicians reported that in comparison to the initial examination, the condition of the wounds had improved.

Discussion

Cutaneous wounds and particularly non-healing wounds pose a considerable therapeutic problem. Despite availability of highly effective surgical techniques it appears that not all patients eligible receive these treatment options. A large proportion of non-healing wounds become contaminated.

While the exact role of wound contamination with micro-organisms is still controversially debated [for a review, see 1], consensus exists that wounds constitute a port of entry for systemic infection such as erysipelas. Nevertheless, it is conceivable that some bacterial products such as *Pseudomonas aeruginosa* exotoxin for example may delay wound healing [9, 10] due to its cytotoxic properties [11].

Antimicrobial wound dressings have gained widespread acceptance in situations where overgrowth of micro-organisms is supposed to delay healing and where the use of systemic antibiotic treatment is not indicated yet. Furthermore, silver-coated textiles were also shown to be effective in other skin diseases such as atopic dermatitis where modulation of the skin flora was demonstrated [12]. Most of the commercially available antimicrobial dressings use silver as active ingredient. Silver ions released from the dressing are bactericidal [4, 13, 14] and resistance is known [15] but still uncommon in the clinical setting [for a review, see 16]. The amount of silver release can be controlled by various means most notably by increasing the surface of the incorporated silver preparation. Nanocrystalline silver dressings have been shown to release high amounts of silver ions over a short period of time [4, 17]. One drawback of free silver ions relates to

their pronounced cellular toxicity [18]. Thus, the balance between antimicrobial activity and cellular toxicity remains a challenge for developing new products which may interfere less with normal wound-healing processes.

We compared a new silver-containing ointment dressing, Atrauman Ag, with established products in this regard. Atrauman Ag was formulated to release as little silver as necessary to safeguard antimicrobial activity while minimising cytotoxic effects. Indeed, the low amounts of released silver were able to confidently kill a panel of micro-organisms which are commonly encountered in non-healing wounds including methicillin-resistant *S. aureus* [19, 20]. Neither the density of the micro-organisms in the test medium nor leaching of silver ions over time diminished the antimicrobial activity. In fact, even 9 days of repeated application of fresh bacteria did not reduce the antimicrobial efficacy. Keratinocytes are normally in close contact with dressings and particularly the migrating epithelial tip may be most susceptible to toxic substances [6]. So, we exposed HaCaT keratinocytes to medium previously incubated with the different dressings. Atrauman Ag showed the lowest cytotoxicity when compared to the other dressings. From these data, nanocrystalline silver results in a fast and strong silver release; however, this is associated with significant cytotoxicity. One may debate whether these high silver levels are indeed needed to limit the growth of micro-organisms or whether wound healing may suffer disproportionately most. Comparing the amounts of released silver with the cytotoxicity data, Actisorb silver 220 appeared to have the lowest silver release while cytotoxicity was slightly higher compared with Atrauman Ag. This observation suggests that Actisorb silver 220 might either release other cytotoxic substances from the dressing or that the activated charcoal component could absorb cytoprotective factors from the medium. The latter mechanism could increase the cytotoxicity of even low amounts of released silver in our experimental setting.

That low amounts of released silver can improve wound healing is further suggested from the clinical observation with Atrauman Ag. The new dressing showed a very good performance profile as from the in vitro data and the judgement of the wound appearance. In an unselected panel of patients, mostly reflecting non-healing wounds encountered in daily practice, the wound condition improved already after 3 consecutive dressing changes. As in this first observational study no control group was selected, a placebo effect cannot be excluded completely. However, no change of clinical routine was requested suggesting that Atrauman Ag was added to the standard therapy which remained almost unchanged. The long duration of the non-healing wounds and the positive assessment by the treating physicians indicate that Atrauman Ag was beneficial, possibly due to the low silver-releasing properties. The effects on the microbiological pattern in the wound bed will be of interest and the subject of further analysis.

We conclude that the new, low silver-releasing ointment dressing reported here has a superior profile of antimicrobial activity over cellular toxicity. This may very well augment tissue repair mechanisms in wounds with delayed healing.

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