DIFERENTIAL CELL DEATH PROGRAMMES INDUCED BY SILVER DRESSINGS IN VITRO

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Introduction

The ultimate goal of wound management and therapy is fast healing and re-epithelialisation with minimal complications. In this way the risk of reduced functionality and aesthetic discomfort greatly diminishes. In order to realize this objective, a great deal of attention is paid to preventing infection of the wound.

One strategy gaining renewed attention for fighting the threat of microbial infection and preventing wound sepsis is the use of silver. Traditionally, two main products have been used for this purpose. Silver-nitrate is active against a variety of micro-organisms and is used in a concentration of 0.5% on patients with extensive burns. Silver sulphadiazine combines the inhibitory action of the silver with the antibacterial effect of sulphadiazine.

In the years following the introduction of these products more concerns rose about safety and disadvantages of the main application occurring during treatment with AgNO₃ was a drop in serum sodium and chlorine due to ion exchange between Ag⁺ and Cl⁻; HCO₃⁻, CO₃²⁻ and protein anions, leading to the production of very slightly soluble or insoluble salt solutions. Secondly, it was observed that during AgNO₃ treatment all objects which came into contact with the AgNO₃, coloured black on exposure to light. Thirdly, elevated silver levels were found in the kidneys, spleen, liver and muscles of two patients on post-mortem examination.

To resolve these problems, wound-care companies searched for improved products combining the strength of silver with technological advances in wound dressings. The new products offer the opportunity of a slow release of silver enabling less frequent changes of dressings. The result is a myriad of Ag-dressings on the market. Although the cytotoxicity of these dressings is reported to be less pronounced, some authors do describe reduced viability of cells after contact with such silver compounds.

In order to assess the antibiosis surrounding the subject, we decided to verify the cytotoxicity of various commercially available Ag-dressings, all profoundly different in both composition and type of silver.

Methods

Cell death analysis in the presence of silver dressings

Cells were seeded subconfluently into 6 well plates and were grown overnight at 37°C/5% CO₂ until 90-100% confluency was reached. Silver dressings were divided into 1.5x1.5 cm dimensions and were weighed. The dressings were placed on transwell inserts and overlaid with 1.2 ml of growth medium. Cells grown in the presence of transwell inserts alone were used as control. The experimental set-up ensured no direct contact between cells and dressings.

Because of this, consequent cytotoxicity can only be the result of diffusion from the dressings under investigation (Fig. 1A). Cells with dressings were incubated at 37°C/5% CO₂ for 4 hours, after which the cells were analysed visually. For cell death analysis the cells were incubated for 2 hours in the same experimental set-up as above. The type of cell death programme was analysed using the Apoptosis Detection Kit (Sigma, Bornem, Belgium) according to the manufacturer’s instructions.

Bioactivity of silver from different dressings

Gram-positive S. aureus and Gram-negative E. coli cells were dispersed in liquid broth (TS) to obtain an optical density of 600 nm (OD₆₀₀) of 0.1. S. aureus cells were re-suspended to obtain an OD₆₀₀ of 0.5. Dressings were dispersed in liquid TS medium at 40 mg/ml for 24h at 37°C / 230 rpm. Afterwards, 1/2 serial dilutions of the ‘extracts’ were made in microtub growth media. One hundred microlitres of the freshly made dilutions was poured into 96 well plates and 2 μl of the microbial suspensions were added to the mixture. Wells with only liquid growth medium served as a positive control for growth. Micro-organisms were allowed to grow for 24h at 37°C. Growth was measured spectrophotometrically at 600nm (MKII, Dynex Technologies, US) and MIC₅₀ values were calculated.

Zone of inhibition

Micro-organisms were suspended in sodium chloride-peptone (OD₆₀₀ = 1) and plated on agar plates. Dressings were divided into pieces of 1 cm x 1 cm and placed directly on top of the agar plates. Plates were incubated for 24 hours at 37°C. After incubation the zone of inhibition surrounding the dressings was measured.

Statistical analysis

The results are the mean and standard deviation of at least 3 independent experiments. The data were analysed by the Student t-test.

Results

Survival of active silver on keratinocytes and fibroblasts in vitro

After 4h of incubation, visual inspection of the cells clearly showed that for all dressings, cells were rounding up or deforming, indicative of a cell being in a stress situation (Fig. 1B). MTT analysis confirmed our visual observations in that the silver dressings significantly influence cell survival compared to control cultures (p<0.01) (Fig. 1C). Both types of analysis demonstrate that all tested silver dressings release their active silver complexes which then diffuse into the surrounding medium thus influencing cell morphology and survival.

Figure 1. Silver dressings influence cell survival by diffusion: A) Graphical illustration of the experimental setup. Dressings were weighed for cell extracts. B) Photograph illustrating the experimental setup, with E.coli cells treated with Ag dressings and negative controls (Sigma, Kontich). C) Cytotoxicity of dressings was measured using the MTT assay.

Analysis of cell death programme induced by the various dressings

After incubation, cells were double stained with Annexin-V-FITC, a marker for early apoptosis (phosphatydilserine exposure to the outer cell surface) and propidium iodide, a marker for necrosis (cell membrane damage) and analysed using flow cytometry. A marked difference in response was seen for the two cell lines (Table 1).

Table 1. Flow cytometric analysis of HaCaT keratinocytes and HUVEC 12SR. Values are means of three experiments expressed as percentage; numbers between brackets represent standard deviations. *p<0.05, **p<0.01.

<table>
<thead>
<tr>
<th>Dressing</th>
<th>Control</th>
<th>HaCaT*</th>
<th>HUVEC**</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO₃</td>
<td>18.75%</td>
<td>10.50%</td>
<td>31.50%</td>
</tr>
<tr>
<td>Actosan¹</td>
<td>6.50%</td>
<td>5.50%</td>
<td>21.50%</td>
</tr>
<tr>
<td>Bactena²</td>
<td>6.50%</td>
<td>5.50%</td>
<td>15.50%</td>
</tr>
<tr>
<td>Bactena³</td>
<td>6.50%</td>
<td>5.50%</td>
<td>15.50%</td>
</tr>
<tr>
<td>SilverAg</td>
<td>6.50%</td>
<td>5.50%</td>
<td>15.50%</td>
</tr>
</tbody>
</table>

Antimicrobial activity of the silver dressings on agar plates to control S. aureus, E. coli and C. albicans suspensions on agar plates were challenged with the different silver dressings on plate. Table 2 shows that the susceptibility of the micro-organisms to the dressings is very different.

Biological effect by dilution

As every dressing has its own density resulting in varying amounts of silver being released per surface area, we decided to suspend a fixed amount of the silver dressings in growth medium for 24 hours and to grow serial dilutions of the “extract”. Next, a fixed number of micro-organisms were challenged with the dilutions for 24h and growth was assessed spectrophotometrically (Table 3).

Table 2. Zone of inhibition measurements in mm. Results are expressed as mean ± SD of three independent experiments.

<table>
<thead>
<tr>
<th>Dressing</th>
<th>Staphylococcus aureus</th>
<th>Candida albicans</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actosan¹</td>
<td>2.00±0.00 (1)</td>
<td>3.87±1.20 (1)</td>
<td>1.58±0.50 (1)</td>
</tr>
<tr>
<td>Bactena²</td>
<td>0.50±0.00 (1)</td>
<td>1.87±0.50 (1)</td>
<td>2.52±0.50 (1)</td>
</tr>
<tr>
<td>Contra²</td>
<td>2.50±0.00 (1)</td>
<td>3.00±0.10 (1)</td>
<td>6.00±0.00 (1)</td>
</tr>
<tr>
<td>SilverAg</td>
<td>0.00±0.00 (1)</td>
<td>0.00±0.00 (1)</td>
<td>0.00±0.00 (1)</td>
</tr>
</tbody>
</table>

Table 3. MIC₅₀ values for dressings extracted for 24h incubated growth media. Results are shown as mean ± SD. Values are shown as mean ± SD of at least three independent experiments expressed at ng/ml. A bacterial growth medium.

Conclusions

Our experiments show that the antimicrobial activity of silver dressings is accompanied by cellular cytoxicity. As our experimental data were performed on time scales which are relatively short compared with in vivo situations, it therefore seems that silver dressings should be used only on critically contaminated wounds rather than used de facto. The search for antimicrobial dressings with good antimicrobial activity but with minimal toxicity towards eukaryotic cells should therefore continue.

**References**

We recommend extensive further studies to confirm our findings and establish guidelines for the use of antimicrobial dressings.