In Vitro Studies to Show Sequestration of Matrix Metalloproteinases by Silver-Containing Wound Care Products

Michael Walker, PhD; Philip G. Bowler, M Phil; and Christine A. Cochrane, PhD

Excess or “uncontrolled” protease activity in the wound bed has been implicated as one factor that may delay or compromise wound healing. One protease group — matrix metalloproteinases — includes collagenases, elastase, and gelatinases and can be endogenous (cell) or exogenous (bacterial) in origin. A study was conducted to assess the ability of five silver-containing wound care products to reduce a known matrix metalloproteinase supernatant concentration in vitro. Four silver-containing wound dressings (a carboxymethyl cellulose, a nanocrystalline, a hydro-alginate, and a collagen/oxidized regenerated cellulose composite dressing), along with a 0.5% aqueous silver nitrate [v/v] solution and controls for matrix metalloproteinase-2 and matrix metalloproteinase-9 sourced from ex vivo dermal tissue and blood monocytes, respectively, were used. Extracts were separated and purified using gelatine-Sepharose column chromatography and dialysis and polyacrylamide gel electrophoretic zymography was used to analyze specific matrix metalloproteinase activity. All dressings and the solution were shown to sequester both matrix metalloproteinases. The silver-containing carboxy-methyl cellulose dressing showed significantly greater sequestration for matrix metalloproteinase-2 at 6 and 24 hours (P <0.001) compared to the other treatments. For matrix metalloproteinase 9, both the carboxy-methyl cellulose dressing and the oxidized regenerated cellulose dressing achieved significant sequestration when compared to the other treatments at 24 hours (P <0.001), which was maintained to 48 hours (P <0.001). Results from this study show that silver-containing dressings are effective in sequestering matrix metalloproteinase-2 and -9 and that this can be achieved without a sacrificial protein (eg, collagen). Although the varying ability of wound dressings to sequester matrix metalloproteinases has been shown in vitro, further in vivo evidence is required to confirm these findings.

KEYWORDS: matrix metalloproteinases, silver-containing dressings, enzyme sequestration

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Dr. Walker and Mr. Bowler disclose they are employees of ConvaTec, UK.
Delayed or compromised wound healing has, in part, been attributed to excess or "uncontrolled" protease activity in the wound bed. This has been supported by in vivo studies in which exudate from chronic leg ulcers and postoperative wound repair data have been analyzed. The family of matrix metalloproteinases (MMPs) comprises mostly zinc-based enzymes and includes generic classes such as collagenases, elastase, and gelatinases. They may be of endogenous (neutrophil or keratinocyte) or exogenous (bacterial) origin. The latter group includes collagenolytic enzymes found in many species, including common wound pathogens (e.g., Pseudomonas aeruginosa and Staphylococcus aureus).

Whatever the source, these enzymes degrade the extracellular matrix, which can lead to tissue destruction and the development of non-healing ulceration if activity is uncontrolled.7

Proteases have been shown in vivo studies to play a critical role in many of the physiologic processes involved in wound repair, including the early stages of clotting and inflammation, and later, clot lysis, fibroplasia, angiogenesis, and extracellular matrix remodeling.8 This proteolytic activity is tightly regulated in acute wounds to ensure a balance exists between tissue synthesis and tissue degradation with control occurring at the cellular and extracellular level (see Figure 1).

Breakdown in one or more of these control mechanisms may result in an increase in proteolytic activity (e.g., by MMPs), which gives rise to immunopathology, a recognized condition in non-healing wounds.9 This is particularly true when exogenous proteases produced by bacterial pathogens are present because these enzymes are not regulated by host inhibitors4 (see Figure 2). Previous in vivo studies have shown increased activity of MMP-2 and MMP-9 in human chronic wound fluid10 and diabetic and venous ulcer tissue.11 Matrix metalloproteinases have been shown to play an important role during tissue repair processes.12 However, if uncontrolled and continually expressed (as occurs in chronic inflammation), it has been shown in pressure ulcer granulation tissue that these proteases provide a destructive proteolytic environment that may inhibit healing and epidermal wound migration.13

![Figure 1. Schematic of "controlled" acute wound healing events.](image)

**KEY POINTS**

- Since it was discovered that the exudate of wounds that fail to heal contains more matrix metalloproteinases (MMPs) than the exudate of wounds that are healing, researchers have tried to understand the implications of these observations.
- If indeed excess protease activity is the cause, not the result, of protracted healing, wound products that absorb and sequester these proteases could help chronic wounds heal.
- In this in vitro study, the authors showed that silver-containing dressings effectively sequester proteases.
- If clinical outcomes using the wound care products evaluated mirror the results observed in this pre-clinical study, the findings reported here may help explain why.
A variety of technological approaches has been applied to wound dressings in an attempt to control MMP activity. Clinical studies have shown that a "sacrificial" protein (e.g., collagen) can quench the high proteinase activity while in other in vitro studies the technological approach relies on the physical absorption and retention of exudate and its biological components. Chronic wound exudate is commonly believed to be a corrosive biological fluid, as such, it is important that this fluid is retained within an applied dressing to reduce the likelihood of further tissue destruction. The use of silver-containing dressings has been reported in in vivo studies to reduce MMP activity although the mechanism for this is, as yet, unclear. One explanation may be the displacement of zinc by silver from the MMP enzyme; the silver ion Ag⁺ has a higher redox potential (E°) than the zinc Zn²⁺ ion (+0.80 and -0.76 v, respectively) but current research to support this theory is lacking.

The purpose of this in vitro study was to assess the ability of silver-containing wound care products (e.g., four dressings and a solution) to sequester MMPs.

**Methods**

**Test materials.** Four silver-containing dressings were evaluated in this study: a silver-containing carboxymethyl cellulose (CMC) Hydrofiber dressing (SHD; AQUACEL® Ag, ConvaTec, a Bristol-Myers Squibb Company, Princeton, NJ); a nanocrystalline silver-containing dressing (NSD; Acticoat® Burn, Smith and Nephew Inc. Largo, Fla); a silver-containing hydro-alginate dressing (SHA; SILVERCEL®, Johnson & Johnson Wound Management, a division of Ethicon Inc, Somerville, NJ), and a silver-containing oxidized regenerated cellulose/collagen composite dressing (SORC; Promogran™ Prisma® Matrix, Johnson & Johnson Wound Management, a division of Ethicon Inc, Somerville, NJ). To assess the effect that silver alone may have on specific MMP activity, a 0.5% aqueous silver nitrate (w/v) solution (SN) also was evaluated.

**Matrix metalloproteinase preparations.**

**Isolation of MMP-9.** Peripheral blood, taken after diagnostic tests had been performed, was collected by venipuncture, anticoagulated with heparin, and refrigerated for 30 minutes. The plasma was then centrifuged at 200 g/4°C for 10 minutes. Supernatant plasma was removed and the pellet was re-suspended in plasma using 10% of the original volume of blood.

Monocytes were isolated using a percoll (Sigma, UK) gradient. Stock percoll was made up using 1 part Hank's balanced salt solution (HBSS; Gibco, UK) and 9 parts percoll. (Note: All cell culture materials were supplied by Gibco, UK unless otherwise stated). Percoll was applied to the bottom of the tube at a concentration of 62.5% to facilitate the separation of monocytes from the plasma. The cell-rich plasma was layered onto the percoll gradient slowly in order to avoid mixing. The tubes were centrifuged at 400 g/4°C for 10 minutes. The white layer above the Percoll-containing monocytes was removed and the cells were washed three times with HBSS. The cells were lysed using 0.1% Triton X-100 and stored at -70°C until required.

**Isolation of MMP-2.** Dermal tissue was obtained post mortem from horses that had been killed for other reasons. Tissue samples were washed in HBSS
Figure 3. MMP-2 sequestration by silver-containing dressings and solution. The control MMP-2 (----) showed no loss of activity over the 48-hour period. * = statistical significance between the SHD and the other treatments at 6 and 24 hours (P < 0.001).

Figure 4. MMP-9 sequestration by silver-containing dressings and solution. The control MMP-9 (----) showed no loss of activity over the 48-hour period. * = statistical significance (P < 0.001) for the SHD and the SORC dressing compared to the other treatments.

and cut into 3-mm³ to 5-mm³ pieces and placed into 25-cm² culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 20 mM Hepes buffer, 100 μg/mL gentamicin, and 0.5 μg/mL amphotericin B (fungizone).

The cultures were incubated in 5% carbon dioxide in air at 37°C. Readiness for sub-culturing was determined by the extent of fibroblast cell outgrowth (5 to 10 days). Cells were frozen successively in a 1:4 split ratio and passages 3 through 8 were used in the experiments. Cultures of fibroblasts were grown to confluence, the media were removed, and the cell layer was washed with HBSS to remove all traces of FCS. The medium was changed to DMEM supplemented with 20 mM Hepes buffer and 1% penicillin-streptomycin. The cells were left to grow to confluence for 5 days, after which the medium was removed and centrifuged at 1,400 rpm for 5 minutes. The supernatant was removed and stored at -70°C until enzyme purification was required.

Enzyme purification. Samples of cell extracts from lysed monocytes and media from confluent fibroblasts were dialyzed in equilibrating/loading buffer (0.05M Tris, 0.5M sodium chloride, 0.005 calcium chloride, 0.05% Brij, 0.02% sodium azide, at pH 7.6) overnight at 4°C. A 5-mL gelatin-Sepharose 48 mm column (Amersham Biosciences, UK) was set up and washed twice with equilibration buffer. A separate column was set up for the isolation of each enzyme (ie, MMP-2 or MMP-9).

Either the cell supernatants or the cell extracts were added to the column, collected in a beaker, and discarded. The column was washed five times with 5 mL equilibrating buffer. The enzyme was eluted from the column by adding 10 mL of 10% dimethyl sulphoxide in 80% elution buffer (0.05 M Tris hydrochloride, 1 M sodium chloride, 0.005 M calcium chloride, 0.05% Brij 35%, 0.02%
sodium azide) and collected in aliquots of 2 mL in bijou bottles. Fractions were dialyzed against MMP buffer (0.05M Tris hydrochloride, 0.005 M calcium chloride, 0.05% Brij 35, 0.02% sodium azide). These aliquots were stored at -70°C until required for use. The columns could be rehydrated in equilibrating buffer and stored at 4°C until required.

Dressing interaction studies. Silver-containing dressing samples (0.1 g) or 10 μl aqueous SN were incubated separately with either purified MMP-2 or MMP-9 (500 μl). Six samples of each dressing and a control were incubated at each time point (i.e., control at 5 minutes [n = 6]) and 1, 3, 6, 24, and 48 hours at 37°C. After incubation, 1 mL PBS was added and the supernatants were removed from the vials and centrifuged at 200 g for 10 minutes at 4°C, then divided into 1-mL samples and frozen at -70°C until required for analysis.

Treatment of samples. Samples were diluted with non-reducing sample buffer (1M Tris hydrochloride pH 6.8, 0.5% sodium dodecyl sulphate [SDS] [w/v], 1.7% glycerol [v/v], 0.05% bromophenol blue [w/v], and distilled water) at a 1:5 dilution. Samples were incubated at 37°C for 1 hour before application to the gel.

Zymography was performed using a Miniprotein II gel electrophoresis system (Biorad, UK). Zymography is an electrophoretic technique based on SDS polyacrylamide gel electrophoresis that includes a substrate co-polymerized with polyacrylamide gel for the detection of MMPs. In this study, gelatin was the substrate used to detect the presence of MMP-2 and -9. A 7.5% resolving gel was made by co-polymerizing acrylamide/bis-acrylamide stock with gelatin. This produced a 0.25% gelatin/7.5% acrylamide gel, which was cast using 3.2 mL of the gel solution in each set of glass plates. Once the gel was set, a stacking gel buffered at pH 6.8 was added. When the stacking gel had set, the samples were loaded and subjected to electrophoresis at a constant 200 v for 45 to 60 minutes or until the dye front reached the bottom of the resolving gel. A set of high molecular weight markers was run on each gel. After electrophoresis, the stacking gel was removed and the marker lane stained in 0.5% Coomassie blue for 30 minutes and then de-stained until the markers became visible. Markers then were washed in water and dried.

The resolving gels were washed in 2.5% Triton X-100 for 1 hour to remove any remaining SDS and then repeatedly rinsed in distilled water. The resolving gels then were placed in gelatin refolding buffer and incubated for 24 hours at 37°C. Following incubation, the gels were placed in 2% Coomassie Blue stain for 30 minutes and de-stained for 60 minutes until the digestion bands became clear. The gels were washed in distilled water for 1 hour and dried between cellophane sheets using a Hoeffer gel drying system.

Gel quantification. Gel images were captured and analyzed using the GeneGenius Gel Documentation System (Syngene, Cambridge, UK). The relative activity value was calculated as follows:

$$RAV = \frac{\text{Peak area of sample}}{\text{Peak area of } +ve \text{ control}}$$

Statistical analysis. Data were statistically analyzed using a one-way analysis of variance (ANOVA) (Minitab Release 14, UK). This analysis was performed to determine whether any significant decrease in protease activity occurred in the wound care products tested in this study. The relative activity was determined as described in the gel quantification section.
Results

Throughout the 48-hour study period, no loss of activity for either enzyme (i.e., MMP-2 or MMP-9) was noted in the absence of any silver-containing wound care product and remained at 100% (see Figures 3 and 4).

All four test dressings and the aqueous SN showed a substantial reduction (>80%) in MMP-2 activity over the 48-hour study period (see Figure 3). A statistically significant difference was noted between the SHD and all other silver-containing wound dressings as well as the solution at 6 hours (P <0.001) and all treatments at 24 hours (P <0.001). With MMP-9, the SHD and SORC were shown to achieve significant reduction in activity by 24 hours (P <0.001), which was maintained to 48 hours (P <0.001) (see Figure 4). The aqueous SN was shown to eliminate MMP-2 activity by 48 hours and to reduce MMP-9 activity by more than 70%. Figures 5 and 6 show zymograms of MMP-2 and MMP-9, respectively.

Discussion

In chronic wounds, silver-containing dressings are utilized primarily to control bacterial populations. However, additional dressing properties are required to support optimal conditions for wound healing. These include the ability to manage exudate and sequester proteases that may be detrimental to wound healing. Exudate management and protease sequestration have been demonstrated through both in vitro and in vivo studies to adversely influence the healing process if not controlled adequately and each is integral to the current systematic approaches to wound management — ie, the evaluation of tissue (non-viable or deficient), the effects of inflammation and/or infection, moisture control and its imbalance, and investigating the edge of the tissue (is it starting to heal or not?). Of the four dressings evaluated, two (SORC and SHD) have been designed to help control both protease sequestration and excessive wound exudate.

Since the in vitro findings by Wysocki et al24 that MMP-2 and MMP-9 were elevated in wound fluid from chronic leg ulcers, much research has been directed at increasing understanding of the mechanisms and roles of MMPs in chronic wounds. This has resulted in the development of dressings specifically designed to reduce protease activity. In particular, the effects of silver on MMP activity have been recognized in a porcine wound model25 and dressings are now available that may modulate MMP activity via several different mechanisms. Reduced MMP activity could be due to either the known binding of silver ions to proteins or to the mechanism through which silver inhibits serine proteases.9 Evidence exists that shows silver complexes with base pairs in DNA26 and with metallothioneins,27 as well as to the prokaryotic protein azurin.30 It is possible that silver protein-binding close to the active site of the MMP enzyme may account for this effect. Silver binding and consequent inhibition have been reported with sodium and potassium ATPases28 and lactate dehydrogenase,29 as well as non-specific binding to enzyme sulphhydryl and disulphide groups.31

<table>
<thead>
<tr>
<th>Table 1</th>
<th>INDENTATION QUALITY (IQ) VALUES IN MM (N = 50)</th>
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<tr>
<td><strong>Mattress Point</strong></td>
<td><strong>Mean ± SD</strong></td>
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<tr>
<td>1 = relatively unloaded zone</td>
<td>-11.91±2.58</td>
</tr>
<tr>
<td>2 = head and heel zone</td>
<td>-13.25±2.36</td>
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<tr>
<td>3 = knee and shoulder zone</td>
<td>-16.68±2.24</td>
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<tr>
<td>4 = buttocks zone</td>
<td>-26.96±4.31</td>
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Note: all IQ values are given as minus values

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<tr>
<th>Table 2</th>
<th>PEARSON CORRELATION COEFFICIENTS BETWEEN INDENTATION QUALITY (IQ) VALUE AT DIFFERENT POINTS ON THE MATTRESS (N = 50)</th>
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<tbody>
<tr>
<td>IQ value</td>
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<tr>
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<td>.70*</td>
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<td>Point 3</td>
<td>.33*</td>
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<tr>
<td>Point 4</td>
<td>.26*</td>
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</table>

* P <0.01, † P <0.05, ‡ P <0.10
In these *in vitro* studies, all the silver-containing wound care products and the silver nitrate solution were shown to reduce MMP-2 and MMP-9 supernatant concentration to varying degrees. In contrast, in the absence of any silver-containing product, no loss of proteinase activity occurred (see Figures 3 and 4). This is in contrast to results from *in vivo* studies presented by Cullen et al., who suggested that a >90% reduction in MMP activity occurred within 24 hours using an oxidized regenerated cellulose/collagen composite dressing. In those studies, the levels of MMP activity in wound fluid taken from diabetic foot ulcers indicated minimal activity was expressed at the outset, so perhaps this result is not surprising. However, in other *in vivo* studies in humans and horses, highly elevated levels of proteinase activity have been shown.

The SHD was shown to reduce proteinase concentrations in the supernatant throughout the 48-hour study period with equal effect on each of the two MMPs studied. Interestingly, SORC tended to reduce MMP-2 concentration to a lesser degree than MMP-9 and consequently statistically significant differences were noted between these two dressings at 6 and 24 hours (P <0.001). In this instance, the addition of silver appears to have had two effects: first, it reduced the overall magnitude of the concentration of MMP-9 and second, it reduced its effect on MMP-2. This effect was not observed with SHD; the results showed that the sequestration of both MMP-2 and MMP-9 was equal and consistent throughout the study period.

These laboratory studies have shown measurable reduction in MMP activity using standard enzyme techniques (eg, polyacrylamide gel electrophoresis) but caution must be exercised in extrapolating these results to the clinical situation. As such, further studies are warranted.

**Conclusions**

All four silver-containing wound dressings tested, as well as the silver nitrate solution, were shown to reduce the supernatant concentration of both MMP-2 and MMP-9 *in vitro*. These findings indicate a quantifiable effect of silver on MMPs. The sacrificial protein in SORC (collagen) was as effective as the silver nitrate solution and the other dressings in reducing the supernatant concentration of MMP-2 and was shown to be equivalent to SHD in reducing the supernatant concentration MMP-9. These *in vitro* studies suggest that 1) MMP down-regulation can be achieved without the need for a sacrificial protein; 2) silver is effective in reducing MMP-2 and MMP-9 concentrations; and 3) SHD is the most effective silver-containing dressing tested in sequestering MMP-2 and MMP-9.

**References**


14. Wysoki AB, Staiano-Coico L, Grinnell R. Wound fluid from chronic leg ulcers contains elevated levels of metallopro-


Run Sheet for Medicel Burns Ag Processing

<table>
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### Silverisation Step (Sprayhead 'A')

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Comments


