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Silver Oxysalts Promote Cutaneous Wound Healing Independent of Infection

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Abstract

Chronic wounds often exist in a heightened state of inflammation whereby excessive inflammatory cells release high levels of proteases and reactive oxygen species (ROS). While low levels of ROS play a fundamental role in the regulation of normal wound healing, their levels need to be tightly regulated to prevent a hostile wound environment resulting from excessive levels of ROS. Infection amplifies the inflammatory response, augmenting levels of ROS which creates additional tissue damage that supports microbial growth. Antimicrobial dressings are used to combat infection; however, the effects of these dressing on the wound environment and healing independent of infection are rarely assessed. Cytotoxic or adverse effects on healing may exacerbate the hostile wound environment and prolong healing. Here we assessed the effect on healing independent of infection of silver oxysalts which produce higher oxidative states of silver (Ag^{2+}/Ag^{3+}). Silver oxysalts had no adverse effect on fibroblast scratch wound closure whilst significantly promoting closure of keratinocyte scratch wounds (34% increase compared to control). Furthermore, dressings containing silver oxysalts accelerated healing of full-thickness incisional wounds in wild-type mice, reducing wound area, promoting re-epithelialisation and dampening inflammation.

We explored the mechanisms by which silver oxysalts promote healing and found that unlike other silver dressings tested, silver oxysalt dressings catalyse the breakdown of
hydrogen peroxide to water and oxygen. In addition, we found that silver oxysalts directly released oxygen when exposed to water. Collectively, these data provide the first indication that silver oxysalts promote healing independent of infection and may regulate oxidative stress within a wound through catalysis of hydrogen peroxide.

**Introduction**

Chronic wounds, which include diabetic, venous and pressure ulcers, are a significant global problem, causing patient morbidity and a substantial financial burden on health services worldwide. The incidence of chronic wounds is currently rising because those populations most susceptible, the elderly and diabetic, are rapidly expanding\(^1\). This in turn puts increasing financial strain on health services. To put this in perspective, while the UK population is estimated to rise just 3.8% in the 5 years from 2014 to 2019; the disproportionate increase in the elderly and diabetic means the incidence of chronic wounds is expected to rise by more than twice as much (9.8%) in the same period\(^1\). In 2014, the annual NHS spend on wound care was estimated at £2 billion\(^2\), while in the U.S. an estimated US$25 billion is spent on their treatment\(^3\). Despite the significant global economic and social impact of chronic wounds, mechanistic understanding of delayed healing remains poor.

Wound infection is a major contributing factor to the development and persistence of chronic non-healing wounds, with wound bioburden (a combination of total bacterial load, species diversity and presence of pathogenic species) linked to healing outcome\(^4\). Most chronic wounds are stalled in the inflammatory phase of healing whereby excessive tissue damage results in prolonged and heightened inflammation. Infection amplifies and
perpetuates this inflammation by recruiting more neutrophils and macrophages. Neutrophils and macrophages release high levels of proteases which cause excessive tissue breakdown. In addition, these inflammatory cells are a major source of reactive oxygen species (ROS; superoxide anion, hydroxyl radicals, $\text{H}_2\text{O}_2$, singlet oxygen). ROS play a fundamental role in the orchestration of normal wound healing. Tissue wounding induces a rapid concentration gradient of $\text{H}_2\text{O}_2$, produced by injured epithelia, which acts as a chemoattractant to guide neutrophils towards injury. The short-lived highly cytotoxic nature of $\text{H}_2\text{O}_2$ is a key mechanism of the innate immune response in the control of infection. However, $\text{H}_2\text{O}_2$ production must be tightly regulated as excess levels damage structural proteins in the extracellular matrix, adversely alter the regulation of signalling pathways and pro-inflammatory cytokines and causes cell death and tissue necrosis. It has been suggested that regulating excessive ROS levels, such as $\text{H}_2\text{O}_2$, in chronic wounds may help reduce the hostile environment and stimulate healing.

The harmful nature of the recruited inflammatory cells and their secreted products causes tissue damage which propagates infection. This cycle leads to a destructive wound environment. Despite the high levels of inflammatory cells present in infected chronic wounds, their phagocytic and bactericidal functions are diminished. As a result the wound becomes more susceptible to an increase in bacterial bioburden.

Antimicrobial therapies are often used to compensate for the inability of the body's innate immune response to control infection in chronic wounds. Dressings incorporating antimicrobial agents such as silver, iodine, honey and polyhexamethylene biguanide (PHMB), are commonly favoured over antibiotics for the treatment of infected wounds,
particularly in the earlier stages of infection\textsuperscript{12,13}. While the effectiveness of antimicrobial wound dressings on bioburden is paramount, their effects on the wound environment must also be considered. Dressings containing singly ionic silver (Ag\textsuperscript{1+}) are arguably the most widely available and frequently used dressing due to their antimicrobial activity and minimal association with microbial resistance\textsuperscript{14,15,16}. However, concerns remain over potential tissue cytotoxicity caused by silver dressings. Any detrimental effects of these antimicrobial dressings on healing may exacerbate the non-healing phenotype observed in chronic wounds. Thus, the effects of silver dressings on healing subsequent to infection clearance, and recommendations for their prophylactic use to prevent infection, remain unclear\textsuperscript{17,18,19,20,21,22}.

New technology can now incorporate silver oxysalts (Ag\textsubscript{7}NO\textsubscript{11}) into dressings which, when exposed to aqueous media such as wound fluid, degrade to produce higher oxidative states of silver (Ag\textsuperscript{2+} and Ag\textsuperscript{3+}). \textit{In vitro} studies have shown these higher oxidative states of silver exhibit greater antimicrobial activity than singly ionic silver (Ag\textsuperscript{1+})\textsuperscript{23,24}. Crucially, the potent antimicrobial activity of Ag\textsuperscript{2+} and Ag\textsuperscript{3+} oxysalts significantly reduces the silver concentration required to achieve bactericidal activity\textsuperscript{23}, reducing the risk of tissue cytotoxicity. However, the effects of silver oxysalt-containing dressings on healing, independent of infection remain unknown. In the current investigation, we demonstrate the safety profile of silver oxysalts using \textit{in vitro} (cell) and \textit{in vivo} (murine wound) studies, while also assessing the potential of silver oxysalts to promote healing independent of infection.
Methods

**Scratch wound assays:** Conditioned media, KerraContact Ag™ (previously Exsalt-T7; Crawford Healthcare, Knutsford, UK) or control dressing (non-woven Sawabond 4383; Sandler, Schwarzenbach/Saale, Germany) was prepared by incubating 10 cm² of each dressing in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM - Sigma-Aldrich, Dorset, England) containing 10% fetal bovine serum (FBS - Sigma-Aldrich, Dorset, England) for 24 h with agitation at room temperature. The control dressing, Sawabond 4383, is composed of the same material as KerraContact Ag, polyethylene and polyester. These materials are biologically inert and have similar absorptive properties. After 24 h media was collected, vigorously shaken, diluted 1 in 10 and applied to scratch wounds as follows. Confluent primary human dermal fibroblasts or human epidermal keratinocytes (HaCaT) cultured in 24-well plates were scratch wounded using the point of a sterile 1000 μl volume pipette tip. One milliliter of silver oxysalt conditioned media, control dressing conditioned media or control media was added to each well immediately after wounding, followed by 24 h incubation at 37 °C under 5% CO₂. Cells were washed in PBS, visualized with crystal violet and imaged using a Nikon Eclipse E600 microscope and SPOT digital camera. Images were blinded and wound closure was calculated using Image ProPlus version 6.1 (MediaCybernetics Inc, Maryland, USA).

**Murine wounding:** All animal work was carried out under a UK Home Office project licences subject to local ethics committee approval and in accordance with the European Guidelines for Animal Care and Use of Experimental Animals. Eight-week-old female C57BL/6 mice (Environ) were anaesthetised and wounded following our established protocol in accordance with Home Office regulations. Briefly, two 1 cm full-thickness incisional wounds...
were made through both skin and panniculus carnosus muscle. A 1 cm² piece of non-woven Sawabond 4383 control dressing (Sandler, Schwarzenbach/Saale, Germany) or KerraContact Ag™ dressing (previously Exsalt-T7; Crawford Healthcare, Knutsford, UK), pre-moistened in sterile distilled H₂O, was placed over each wound. Dressings were then covered with Tegaderm (3M, Bracknell, UK) attached via Mastisol liquid adhesive (Eloquest Healthcare, Michigan, US). Mice were house individually to prevent damage to the wounds and dressings. Three and 7 d post-wounding mice were sacrificed (schedule 1 method), each wound was excised, bisected and processed for histology, allowing the midpoint of the wound to be compared between groups. Ten wounds (five mice) for each treatment group at each time-point were analysed.

**Histological and immunohistochemical analysis:** Histological sections were prepared from wound tissue fixed in 10% buffered formalin saline and embedded in paraffin. Five-micrometre sections were stained with haematoxylin and eosin or subjected to immunohistochemistry with rat anti-Ly6G (neutrophils), rat anti-Mac-3 (macrophage) (BD Biosciences, Pharmentgen, Oxford, UK), anti-keratin 14, anti-keratin 1, anti-keratin 6 and anti-loricrin (Biolegend, San Diego, USA) and the appropriate biotinylated secondary antibody followed by ABC-peroxidase reagent (Vector Laboratories, UK) with NovaRed substrate or streptavidin-Cy3 and counterstaining with haematoxylin or DAPI. Slides were blinded before images captured with a Nikon Eclipse E600 microscope and SPOT digital camera. Total inflammatory cell numbers, re-epithelialisation and wound area were quantified with Image Pro Plus software (MediaCybernetics, Maryland, USA) as previously described²⁵. Keratin 14, keratin 1 and keratin 6 were quantified using Image Pro Plus software (MediaCybernetics, Maryland, USA) as area of expression within the wound margins. Loricrin expression was
Assessment of oxygen generation from silver compounds: One hundred milligrams of silver compounds were added to 20ml of foetal bovine serum and oxygen levels in solution measured using a Hach TQ40D Oxygen probe and meter and levels recorded every minute for 5 min. This meter detects dissolved oxygen in the range 0.00-22.00 ppm. The operating principle of the meter is highly specific to oxygen and is not affected by metal ions or oxidising agents per se.

Assessment of H$_2$O$_2$ breakdown: One hundred milligrams of silver compound were added to 20ml of foetal bovine serum, vigorously mixed before adding H$_2$O$_2$ to a final concentration of 0.08% w/w. Oxygen levels were monitored using a Hach TQ40D Oxygen probe and meter and levels recorded every minute for 5 min. To assess the ability of silver-containing wound dressings to catalyse the breakdown of H$_2$O$_2$, the oxygen level of deionised water containing 0.08% w/w H$_2$O$_2$ was recorded immediately prior to the addition of a single 2x2 cm$^2$ of dressing and at each minute for 5 min following the addition. The procedure was repeated in triplicate.

Statistical Analysis: Statistical analysis was performed on all quantitative data and power calculations performed as previously described$^{26}$. Our statistical power calculations based on a large experimental data set indicate that an inter-group difference of 20% or greater can be reliably detected using 8 wounds. All graphs represent mean +/- standard error. In vitro scratch wound assays, in vivo wound area and inflammatory cell counts were analysed.
with a Mann-Whitney U test. Re-epithelialisation was analysed using an unpaired t-test with Welch's correction. For all statistical tests, probability values of $P$ less than 0.05 were considered statistically significant.

Results

We investigated the effects of silver oxysalts on healing independent of infection in vitro and in vivo. We first assessed the effects of a dressing containing silver oxysalts on healing of fibroblast and keratinocyte scratch wounds in vitro. Specifically, confluent layers of fibroblasts or keratinocytes were scratched and treated for 24 h with control media, media conditioned with a control dressing or silver oxysalt conditioned media (Figure 1). We found no difference ($P=0.53$) in the closure rate of fibroblast scratch wounds treated with silver oxysalt media versus the controls (Figure 1 A-C). By contrast, keratinocyte scratch wounds treated with silver oxysalt media showed significantly ($P=0.04$) accelerated scratch wound closure when compared to control media (Figure 1 D-F).

Next, we evaluated the effects of silver oxysalts on wound healing in vivo utilising a murine, full-thickness, incisional wound model. Upon wounding, mice were treated with a control dressing or a dressing containing silver oxysalts. At both 3 and 7 d post-wounding, silver oxysalt treated wounds appeared visually smaller than control treated wounds (Figure 2 A-D). Argyria, a grey-slate discolouration of the skin following topical or systemic administration of silver compounds was not observed with silver oxysalt treatment. Histological analysis (Figure 2 E-H) and quantification of wound area from histological sections (Figure 2 I) confirmed silver oxysalt-treated wounds were smaller than control treated wounds at 3 and 7 d post-wounding ($P=0.03$ and $P=0.02$, respectively). We next
quantified the extent of re-epithelisation. At 3 d post-wounding silver oxysalt-treated wounds showed significantly advanced re-epithelialisation compared to controls (P=0.05). After 7 d both control and silver oxysalt-treated wounds were fully re-epithelialised. To evaluate restoration of normal epidermal organisation and differentiation, we profiled epidermal differentiation markers 7 d post-wounding (Figure 3). Similar expression profiles of the basal epidermal marker, keratin 14 (Figure 3 A & B), suprabasal epidermal marker, keratin 1 (Figure 3 C & D), and injury induced keratin 6 (Figure 3 E & F) were observed in control and silver oxysalt-treated wounds. Quantification of these keratin markers revealed a non-significant difference in area of expression within the wound margins (P>0.05). We next examined loricrin expression, a marker of late terminal differentiation. In control treated wounds, patchy loricrin expression was observed across the newly re-epithelialised epidermis (Figure 3 G). By contrast, a continuous layer of loricrin expression was observed covering the newly re-epithelialised epidermis in silver oxysalt treated wounds (Figure 3 H). Quantification of percentage loricrin expression confirmed significantly greater coverage in silver oxysalt treated wounds compared to control treated wounds (85% compared to 63%, respectively, P=0.02).

To determine the effects of silver oxysalts on inflammation in vivo we immunohistochemically quantified the infiltration of neutrophils and macrophages into murine granulation tissue (Figure 4). At 3 and 7 d post-wounding silver oxysalt treated wounds exhibited significantly fewer neutrophils (P=0.015 and P=0.03, respectively; Figure 4 A-E) and macrophages (P=0.03; Figure 4 F-J) compared to control treated wounds.
When exposed to aqueous media, the breakdown of silver oxysalts to generate higher oxidative states of silver is proposed to release oxygen\textsuperscript{28}. We therefore tested the ability of silver compounds to directly generate oxygen when exposed to 100% foetal bovine serum. Addition of silver oxysalts to foetal bovine serum generated oxygen; a property not exhibited by any other silver-based compound tested (Figure 5A).

Silver is known to catalyse the breakdown of H\textsubscript{2}O\textsubscript{2} to oxygen and water\textsuperscript{29}. Therefore, we assessed the ability of silver compounds commonly used in wound dressings to catalyse the breakdown of H\textsubscript{2}O\textsubscript{2} to oxygen and water by measuring oxygen levels in silver solutions made up in 100% foetal bovine serum after the addition of H\textsubscript{2}O\textsubscript{2}. Oxygen levels increased after the addition of H\textsubscript{2}O\textsubscript{2} to solutions of silver oxysalts, silver oxides, silver phosphate and silver metal (Figure 5B). We noted elevated oxygen concentrations in the absence of H\textsubscript{2}O\textsubscript{2} at time zero with silver oxysalts, confirming that silver oxysalts also evolve oxygen in the absence of H\textsubscript{2}O\textsubscript{2} (data not shown). We then assessed the ability of commercially available wound dressings containing various silver compounds to catalyse the breakdown of H\textsubscript{2}O\textsubscript{2} by measuring oxygen levels in foetal bovine serum (plus dressings) after the addition of H\textsubscript{2}O\textsubscript{2}. Of all the dressings tested, increased oxygen levels compared to the control were only detected with dressings containing silver oxysalts (KerraContact Ag and KerraCel Ag) indicating that silver oxysalt-containing dressings alone could catalyse the breakdown of H\textsubscript{2}O\textsubscript{2} to oxygen and water (Figure 5C). The release of oxygen through the catalytic breakdown of H\textsubscript{2}O\textsubscript{2} by dressings containing silver oxysalts was visualised as bubbles in a solution of H\textsubscript{2}O\textsubscript{2}, this was unique to silver oxysalt dressings and not observed by dressings containing other silver compounds (Figure 5D).
Discussion

There is a wealth of literature assessing the efficacy of antimicrobial wound dressings; however, few studies determine the impact of these dressings on healing independent of infection. Adverse effects that may exacerbate the already hostile environment of a chronic wound need to be determined to ensure antimicrobial treatment is given appropriately. Our results show that silver oxysalt treatment promotes closure of *in vitro* keratinocyte scratch wounds. Furthermore, we found that dressings containing silver oxysalts accelerated healing of uninfected full-thickness murine wounds, reducing wound area, promoting re-epithelialisation and dampening inflammation. We explored the possible mechanisms by which silver oxysalts promote healing and found they release oxygen directly when exposed to aqueous media and also catalyse the breakdown of hydrogen peroxide to oxygen and water, a property that was unique to dressings containing silver oxysalts.

Silver is becoming increasingly popular as a topical antimicrobial, and a wide range of silver dressings are available which incorporate silver in various formulations. An ongoing concern with the use of silver dressings to treat infected chronic wounds is potential local or systemic cytotoxicity, which may be detrimental to healing. Unproven *in situ*, this concern arises from extrapolation of *in vitro* results, where silver induces cell death in culture. *In vivo*, the effects of silver on healing independent of infection remain controversial; with studies reporting both adverse and beneficial outcomes. The higher oxidative states of silver in silver oxysalts exhibit enhanced antimicrobial action compared to other available silver technologies. We have assessed the effects of silver oxysalt-containing dressings on healing independent of infection. Given the potent antimicrobial action of silver oxysalts we were surprised to demonstrate no adverse effects of silver oxysalts on healing of *in vitro*
fibroblast scratch wounds and an unexpected acceleration of healing of in vitro keratinocyte scratch wounds. Accelerated healing was also observed with silver oxysalt treatment of full-thickness murine incisional wounds, linked to reduced local inflammation and increased re-epithelialisation. These data contrast with that of Burd and co-workers (2007) who reported impaired re-epithelialisation in an ex vivo porcine and an in vivo murine wound model when treated with several dressings containing single ionic (Ag\(^{1+}\)) silver. These conflicting observations could simply reflect the differing models employed, however, the differing effects may be due to the differences in the silver technology.

A number of factors can delay wound healing which can lead to the development of a chronic wound; these include chronic disease, ageing, diabetes, vascular insufficiency, pressure, oedema and infection. Despite the underlying cause, many chronic wounds progress in a similar way, whereby tissue damage results in heightened and prolonged inflammation. This is characterised by excess neutrophil and macrophage infiltration and elevated levels of destructive proteinases and reactive oxygen species such as H\(_2\)O\(_2\). We explored the possible mechanisms by which silver oxysalts promote healing and found that dressings containing silver oxysalts catalysed the breakdown of H\(_2\)O\(_2\) to water and oxygen. This catalysis of H\(_2\)O\(_2\) was unique to silver oxysalt dressings and was not observed with any other silver dressing. During wound healing low levels of H\(_2\)O\(_2\) are essential for effective repair; however, too much adversely affects healing and prolongs the inflammatory phase\(^{32,33}\). Tissue wounding induces a rapid concentration gradient of H\(_2\)O\(_2\) from epithelial cells which signals to recruit inflammatory cells to the site of injury\(^34\). Inhibition of this H\(_2\)O\(_2\) signal is sufficient to impair leukocyte recruitment to the wound\(^34\). It is hypothesised that
$\text{H}_2\text{O}_2$ generation by epithelial cells has two essential functions: killing of bacteria and recruitment of leukocytes. In addition, the recruited inflammatory cells also release $\text{H}_2\text{O}_2$ which is essential for microbial killing; however, at high concentrations $\text{H}_2\text{O}_2$ causes tissue damage and contributes to chronic inflammation$^{32,33,35}$. Thus, in chronic wounds excessive amounts of $\text{H}_2\text{O}_2$ contribute to the delay in healing$^{35,7}$. This has also been shown in vivo whereby the addition of low levels of $\text{H}_2\text{O}_2$ (10 mM) to wounds in diabetic mice promotes healing whereas the addition of higher concentrations of $\text{H}_2\text{O}_2$ (166 mM) retards healing$^{32}$. It has therefore been proposed that interventions to alter $\text{H}_2\text{O}_2$ concentrations within a wound could be a successful therapeutic strategy to reduce the hostile wound environment. While this does not address the underlying cause of delayed healing it may be sufficient to promote healing of chronic wounds$^7$.

A hallmark of non-healing chronic wounds is excessive and prolonged inflammation, a direct causative factor in healing delay. Wounds treated with silver oxysalt-containing dressings showed reduced numbers of macrophage and neutrophils. Depletion of either or both neutrophils and macrophage has been shown to enhance the rate of wound repair and decrease scarring and it has therefore been suggested that modulating leukocyte recruitment may also therapeutically benefit healing of chronic wounds$^{37}$. However, in an infected wound, a reduction in these cells may delay the wounds ability to combat the infection. The potent antimicrobial action of silver oxysalts is expected to compensate for the reduction in inflammatory cell recruitment. Furthermore, the ability of the silver oxysalts to catalyse the breakdown of $\text{H}_2\text{O}_2$ may enhance the innate immune response. The antimicrobial action of $\text{H}_2\text{O}_2$ released from inflammatory cells within the wound relates not
only to its oxidising properties but also to its breakdown to water, releasing various oxygen species and free radicals\textsuperscript{38}. These compounds are more reactive than H\textsubscript{2}O\textsubscript{2} but are short lived and therefore only those radicals generated in the immediate vicinity of microbial cells are likely to have an antimicrobial effect. The presence of a catalyst has been shown to dramatically enhance the killing properties of H\textsubscript{2}O\textsubscript{2} against bacterial biofilms\textsuperscript{39}. Moreover, residual biofilm bacteria are unaffected by antimicrobial treatments unless a catalyst is included and the strength of bacterial attachment to a test surface is significantly loosened by the inclusion of a catalyst\textsuperscript{39}. Thus, in an infected chronic wound silver oxysalts acting as a catalyst at the site of infection may enhance the killing properties of H\textsubscript{2}O\textsubscript{2} through its breakdown to more reactive antimicrobial species.

Another mechanism by which silver oxysalts could promote healing is its ability to increase tissue oxygen levels within the wound. We tested the ability of a variety of silver compounds to directly generate oxygen when added to foetal bovine serum and found that only silver oxysalts can directly generate oxygen. Oxygen plays a critical role in cell function and survival and is required in high demand by many of the cells essential for effective wound repair. Systemic and topical oxygen therapies have been reported to promote healing; however, the mechanisms by which they do so are still being elucidated and these mechanisms are likely to differ between systemic and topical treatments. Positive effects on neo-angiogenesis, collagen formation, re-epithelialisation and antimicrobial activity have been reported\textsuperscript{40}. Although oxygen is critical for effective wound repair, the main contraindication to oxygen therapy for treatment of chronic wounds centres on the role of hypoxia in promoting repair. Upon wounding oxygen levels drop until a state of hypoxia is reached. Hypoxic conditions within a wound induce a tightly regulated response pathway to
modulate cellular functions and ultimately restore oxygen supply via the upregulation of the transcription factor hypoxia-inducible factor-1 (HIF-1). In states of prolonged hypoxia, such as those found in chronic wounds HIF-1 activity is repressed and thus the key signalling pathways are not stimulated. The optimal oxygen concentration for effective wound repair is unclear, too much or too little may impede healing. Thus, adequate wound tissue oxygen is required but may not be sufficient to positively influence healing. Oxygen therapy may therefore only benefit those cases where hypoxia is the only rate limiting factor and all other essential factors are functional. The level of oxygen released during silver oxysalts to silver ion conversion is low; however, direct oxygen release and oxygen produced through the breakdown of H$_2$O$_2$, together with a reduction in oxygen consuming leukocytes may sufficiently increase tissue oxygen levels to stimulate healing of silver oxysalt treated wounds.

Infected non-healing chronic wounds are clinically challenging, requiring antimicrobial treatment to combat infection and methods to reduce the hostile wound environment. There are an increasing number of antimicrobial therapies available for the treatment of infected wounds and whilst many of these are effective at combatting infection, their effects on healing independent of infection are often unknown. Delineating the effects of antimicrobial agents on healing independent of infection is therefore essential to guide clinical protocols for the treatment of infected wounds. The ability of silver oxysalts to combat infection whilst promoting multiple aspects of healing in vivo offer clinicians a novel alternative to currently available antimicrobial dressings.
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Conflict of Interest

Helen Thomason, Jodie Lovett and Christian Stephenson are employees of Crawford Healthcare. Andrew McBain and Matthew Hardman received KTP funding from Innovate UK and Crawford Healthcare.

References


Figure legends

Figure 1. Silver Oxysalts influence human fibroblast and keratinocyte scratch wound closure. (A & B) Representative images of human fibroblast scratch wounds treated with control (A) or silver oxysalt-containing (B) media for 24 h. (C) Quantification revealed no
difference in wound closure between scratch wounds treated with control media or control
dressing conditioned media and those incubated with silver oxysalt media. (D & E)
Representative images of human keratinocyte (HaCaT) scratch wounds treated with control
(D) or silver oxysalt (E) media. (F) After 24 h scratch wounds treated with media incubated
with silver oxysalt media showed a significant increase in healing compared to control media
or control dressing conditioned media treated wounds (n=3). *, P <0.05. Scale bars = 500 µm
(A, B, D, E).

Figure 2. Silver oxysalts promote healing of non-infected mouse wounds. Representative
macroscopic (A-D) and histological (E-H) images of incisional wounds treated for 3 d (A-B, E-
F) or 7 d (C-D, G-H) with control dressing (A, C, E, G) or dressing containing silver oxysalts (B,
D, F, H). (I-J) Quantification revealed significantly reduced wound area following silver
oxysalt treatment compared to control treated wounds at 3 d and 7 d post-wounding (I) and
increased re-epithelialisation at 3 d post wounding (J). n=5 mice (10 wounds). Arrows in E-H
indicate wound margins. * indicates P= <0.05. Scale bars = 200 µm (A-H).

Figure 3. Silver oxysalts promote restoration of epidermal differentiation.
Immunofluorescence staining (red) for keratin 14 (A & B), keratin 1 (C & D), loricrin (E & F)
and keratin 6 (G & H) in murine wounds treated with control or silver oxysalt dressings for 7
d. Silver oxysalt treated wounds exhibited an advanced re-establishment of normal terminal
differentiation compared to control treated wounds. Similar expression profiles of K14 (A &
B) and K1 (C & D) were observed with control (A & C) and silver oxysalt treatment (B & D).
Keratin 6 expression, which is up-regulated in the epidermis upon wounding, was reduced in
silver oxysalt treated wounds (F) compared to control treated wounds (E). In control treated
wounds loricrin expression was patchy (G) whereas a continuous layer was observed in silver oxysalt treated wounds (H). n=5 mice (10 wounds). Scale bars = 50 µm (A-H).

**Figure 4.** Silver oxysalts reduce wound neutrophil and macrophage infiltration. (A-D) Representative neutrophil immunohistochemistry images of control (A & C) and silver oxysalt treated (B & D) wounds for 3 d (A & B) and 7 d (C & D). (E) Quantification revealed significantly fewer neutrophils in wounds treated with silver oxysalts after 3 and 7 d versus control. (F-I) Representative macrophage immunohistochemistry images of control (F & H) and silver oxysalt treated (G & I) wounds for 3 d (F & G) and 7 d (H & I). (J) Quantification revealed significantly fewer macrophages in wounds treated with silver oxysalts after 3 d and 7 d versus control. n=5 mice (10 wounds). * indicates P <0.05. Scale bar = 50 µm (A-D, F-I).

**Figure 5.** Oxygen generation and catalytic oxygen release from \( \text{H}_2\text{O}_2 \) breakdown by silver oxysalts. (A) Oxygen evolution from silver salt powders in foetal bovine serum was monitored over 5 minutes. Dissolved oxygen concentrations increased immediately after addition of silver oxysalts. This behaviour was not observed for any other silver material tested, including silver oxides. (B) Catalytic breakdown of \( \text{H}_2\text{O}_2 \) solution to oxygen by a range of silver salts. Oxygen levels are shown one minute after the addition of \( \text{H}_2\text{O}_2 \). Catalytic breakdown of \( \text{H}_2\text{O}_2 \) to oxygen in foetal bovine serum is achieved by a subset of silver compounds uncommon in wound care and silver oxysalts. Silver compounds commonly used in wound care, including chloride (Aquacel Ag), sulfate (Mepilex Ag) and sulfadiazine (Allevyn Ag) are inactive. (C) Catalytic breakdown of \( \text{H}_2\text{O}_2 \) solution to oxygen by a range of silver wound dressings in foetal bovine serum. Only dressings containing silver oxysalts...
(KerraContact Ag and KerraCel Ag) were found to catalyse the breakdown of H₂O₂ to oxygen and water. (D) Oxygen released through catalytic breakdown of H₂O₂ by silver oxysalt dressings (KerraCel Ag) was visualised as bubbles in a solution of H₂O₂. Bubbles were not observed by dressings containing other silver compounds.
Figure 1. – Colour image
Figure 2. Colour image
Figure 3. Colour image
Figure 4.

Control Ag Oxysalts

Neutrophils 3 days
C D

Macrophage 3 days
H I

Control Ag Oxysalts

Days post-wounding

Neutrophils (mm²)

Macrophage (mm²)

3 7

3 Days post-wounding

Control Ag Oxysalts

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Figure 5.