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An *in vitro* test of the efficacy of silver-containing wound dressings against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in simulated wound fluid☆

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ABSTRACT

An isothermal microcalorimetric assay was used to quantify the efficacy of a silver-containing wound dressing against two common wound pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The growth patterns of the two species were unique and varied depending on the environment in which the organisms were grown. Addition of non-silver-containing dressing altered the growth kinetics while addition of silver (contained either in a dressing or as $AgNO_3$ solution) was seen to elicit inhibition and/or kill depending on concentration. Tests were conducted in nutrient broth and simulated wound fluid. It was found that minimum inhibitory and minimum bactericidal concentration values were higher in simulated wound fluid and under anaerobic conditions. Bioavailability of silver from the wound dressing was 35% against *S. aureus* in nutrient broth and 68% against both species in simulated wound fluid. The data highlight the importance of developing and conducting *in vitro* assays in biorelevant media.

1. Introduction

Healing of cutaneous wounds is a complicated process, influenced by a number of factors. Healing may be hampered by underlying conditions or pathology including arterial insufficiency or vascular disease, such as seen with diabetic foot ulcers (Kranke et al., 2012). While endogenous pathophysiological factors, such as chronic inflammation, are recognised to be important features of chronic wounds, it is widely accepted that microorganisms also play an important role (Bowler, 2002). The mere existence of a chronic wound implies bacterial contamination; if progression to permanent colonisation occurs and the bacterial load increases, wound healing may be delayed as microorganisms overcome the host's defences and invade into deeper tissues, resulting in further damage (Landis, 2008). Progression to infection may also be aided by other factors including poor blood supply to the wound and intrinsic virulence properties of the invading organisms (Siddiqui and Bernstein, 2010; Bowler et al., 2001). Microorganisms involved in wound pathology may originate from either exogenous sources, including from the environment or those introduced by injury, or be endogenous to the surrounding skin and mucous membranes (Siddiqui and Bernstein, 2010). The understanding of the influence of microorganisms in delaying wound healing dates back to the 1960s when Bendy et al. (1964) reported that healing in decubitus ulcers only proceeded when the bacterial load in wound fluid was less than 10^6 cfu/mL.

It is estimated that 1–2% of the populations in developed countries will at some point suffer from a chronic wound and that global expenditure in treating these conditions is $13–15 billion annually (Siddiqui and Bernstein, 2010). Measures taken to reduce the bacterial load in chronic wounds may, therefore, be a useful and attractive strategy in reducing this burden. Silver, in particular, has found particular application in medicated wound dressings, as it shows broad antimicrobial (against both Gram-negative and Gram-positive organisms, Miraftab et al., 2014) and anti-fungal activity (Bowler et al., 2005), although there is debate as to the specific efficacy of silver (Aziz et al., 2012; White and Cutting, 2006) and to its potential toxicity (Hermans, 2006). Understanding the action of silver is complicated by the difficulties inherent in making quantitative measurements *in vivo*, which leaves *in vitro* measurements as

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the best analytical option. As a minimum outcome, development of standard *in vitro* methods will allow quantitative comparison of the efficacy of different products (Ip et al., 2006), while confidence and correlation with *in vivo* performance will develop as the *in vitro* tests become more representative of the chronic wound environment.

A number of standard microbiological tests that can be applied to wound dressings are available, including disc diffusion tests, broth culture tests and viability testing, such as the Live/Dead Baclight™ system (Boonkaew et al., 2013). We argued previously (O'Neill et al., 2003; Gaisford et al., 2009) that the use of isothermal microcalorimetry (IMC) offers many benefits, not least of which is the opportunity to monitor growth of live organisms directly in heterogeneous media containing the actual wound dressing. We demonstrated the efficacy of a commercial product (AQUACEL® Ag, a silver-containing Hydrofiber® dressing) against *Pseudomonas aeruginosa* and quantified the amount of bioavailable silver in the dressing (*ca.* 25%) by reference to a silver nitrate titration. However, the test was developed in a medium optimised to support growth of *P. aeruginosa* and no other challenge organisms were tested. Here, we use the test to evaluate the efficacy of silver-containing dressings against two species (*P. aeruginosa* and *Staphylococcus aureus*) in simulated wound fluid (SWF), in order to increase the *in vitro–in vivo* correlation (IVIVC) of the test. Classical microbiological techniques were employed to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

2. Material and methods

The challenge organisms (*P. aeruginosa*, NCIMB 8628 and *S. aureus*, NCIMB 9518) were grown overnight in nutrient broth (NB; Oxoid Ltd.) for 16 h at 37 °C. Cells were then harvested, washed in phosphate buffered saline (PBS), resuspended in 15% (v/v) glycerol at an organism density of 10^8 cfu/ml and frozen in aliquots (1 mL) over liquid nitrogen (Beezer et al., 1976; Cosgrove, 1979). Aliquots were stored under liquid nitrogen until required. Previous experience (data not shown) has indicated that organisms can be stored for over 6 years in this frozen state and remain viable post thawing with less than 1% decrease in viability. Wound dressings, AQUACEL® (AH) or AQUACEL® Ag (AAgH), were supplied by ConvaTec Ltd. The wound dressings, both comprised of sodium carboxymethylcellulose fibres, differ in that the former has no antimicrobial agent while the latter contains ionic silver. Silver nitrate solution ($AgNO_3$, 0.01 M standard solution) was purchased from Riedel-de Haën. Simulated wound fluid was prepared by mixing maximum recovery diluent (MRD; Oxoid Ltd.) with foetal bovine serum (FBS; Invitrogen Ltd.) in equal volumes.

An aliquot of frozen organism was thawed by immersion in a water bath (40 °C) for 3 min, followed by a period of vortexing (1 min). SWF (2.97 mL), pre-warmed to 37 °C was pipetted into the calorimetric ampoule (glass, 3 mL volume). Thawed aliquots of bacteria, used in each experiment, were subcultured to eliminate the possibility of them having been contaminated and to demonstrate stock uniformity. An appropriate mass (see Section 3) of wound dressing or volume of silver nitrate solution was added to the ampoule and the medium was inoculated with bacterial suspension (0.03 mL), giving a final organism density of 10^6 cfu/mL (selected because this is the minimum concentration at which growth is detectable in our instrument; it would, for instance, have been possible to inoculate to a lower concentration, but this would simply have added a time-lag to the data while the culture multiplied to a concentration of 10^6 cfu/mL). Ampoules were sealed with a crimped metal lid (an air-tight seal being ensured with a rubber disc), vortexed for 10 s, transferred to the calorimeter and allowed to reach thermal equilibrium. Data were recorded with a 2277 Thermal Activity Monitor (TAM; TA Instruments Ltd., UK) operated at 37 °C. Data capture was initiated exactly 30 min post-inoculation with the dedicated software package Digitam 4.1 (1 data point every 10 s, amplifier setting 300 μW). The instrument was calibrated periodically by the electrical substitution method. Data were analysed using Origin 8.1 (Microcal Software Inc.).

MICs of silver nitrate were determined in SWF or NB by a microdilution method in 96-well plates with $AgNO_3$ concentrations ranging from 1×10^{-3} M to 9.8×10^{-7} M, an inoculum of 1×10^6 cfu/mL and in a total volume of 200 μL. MIC values were evaluated after 16 h incubation at 37 °C.

The minimum mass of wound dressing required to inhibit growth was also determined in SWF with an inoculum of 1×10^6 cfu/mL. Masses of AAgH tested were 1, 2.5, 5, 10, 15, 20 and 25 mg. Minimum bactericidal masses were also recorded as the lowest concentration or mass required, respectively, to give rise to 99.9% kill after plating out 10 μL of sample onto iso-sensitest Agar (Oxoid) and incubation at 37 °C overnight (16 h).

Growth curves for comparison with calorimetric data were obtained by inoculating NB (50 mL) with *S. aureus* to a final population of 1×10^6 cfu/mL. The suspension was dispensed (3 mL) into calorimetric ampoules (11). The ampoules were hermetically sealed as described above. One ampoule was used for TAM analysis and the remaining ten were incubated at 37 °C. At two-hour intervals, an ampoule was vortexed before the seal was broken. Optical density readings were recorded with a spectrophotometer (600 nm, Heliosα, Thermo Scientific) and cfu counts were determined by serially diluting and spread plating onto iso-sensitest agar. Colonies were counted following 16 h incubation at 37 °C.

3. Results and discussion

The use of IMC for monitoring bacterial growth has been long established and discussed (Beezer, 1980; Braissant et al., 2010). We have chosen to plot the data as cumulative heat curves, since these most closely resemble the growth curves produced by classical techniques (Von Ah et al., 2009), so we provide first some interpretation of the data. The output from the calorimeter is a plot of power (μW, or $\mu J\,s^{-1}$) as a function of time (t). It follows that the area under the curve is equal to the heat released (μJ). Fig. 1[D] shows the typical power–time trace for *S. aureus* inoculated into NB. It is apparent that the calorimetric trace is complex, comprising a series of peaks and troughs that correspond to different phases of microbial growth. Assignment of those phases can be made by reference to growth curves determined by optical density (OD) or cell number measurements (Fig. 1[A] and [B]). Biphasic exponential growth occurs initially (0–6 h), followed by a stationary phase (6–14 h) before commencement of cell death. Because experiments are conducted in hermetically sealed ampoules, with a small head-space, it is assumed that the initial exponential phase (*ca.* 1 h) represents aerobic metabolism, after which the oxygen in the ampoule is exhausted and the bacteria switch to anaerobic metabolism, resulting in the second exponential phase (*ca.* 5 h). These growth phases are seen in the calorimeter as exothermic peaks over the same time period. Interestingly, while the OD and cell numbers remain constant during the stationary phase, the calorimeter records a broad, increasing exotherm over the same period. Since cell numbers do not increase during the stationary phase, this power must be associated with the bacteria utilising an increasingly diverse range of nutrients. If cumulative heat is plotted with time, Fig. 1[C], biphasic exponential growth is clearly evident prior to the stationary phase (although the gradient of the stationary phase is not zero because of the effect of the exothermic heat measured during this phase).