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Reactive oxygen: a novel antimicrobial mechanism for targeting biofilm-associated infection

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Highlights

- Reactive oxygen species (ROS) delivered by engineered honey or gel.
- Novel antimicrobial with activity against all bacteria as well as antifungal and antiviral activity.
- Topical treatment with antibiofilm activity.
- Therapeutic implications for wound healing and possibly mucosal infection in respiratory and urinary tract.
- Topical and local applications but could be applied to internal mucosal structures.

ABSTRACT

Reactive oxygen species (ROS) is a novel therapeutic strategy for topical or local application to wounds, mucosa or internal structures where there may be heavy bacterial bioburden with biofilm and chronic inflammation. Bacterial biofilms are a significant problem in clinical settings owing to their increased tolerance towards conventionally prescribed antibiotics and their propensity for selection of further antibacterial resistance. There is therefore a pressing need for the development of alternative therapeutic strategies that can improve antibiotic efficacy towards biofilms. ROS has been successful in treating chronic wounds and in clearing multidrug-resistant organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), and carbapenemase-producing isolates from wounds and vascular line sites. There is significant antifungal activity of ROS against planktonic and biofilm forms. Nebulised ROS has been evaluated in limited subjects to assess reductions in bioburden in chronically colonised respiratory tracts. The antibiofilm activity of ROS could have great implications for the treatment of a variety of persistent respiratory conditions. Use of ROS on internal prosthetic devices shows promise. A variety of novel delivery mechanisms are being developed to apply ROS activity to different anatomical sites.

Keywords:

Reactive oxygen species

Biofilm

Prosthetic device infection

Cystic fibrosis

1. Introduction

The overprescription and excessive consumption of antimicrobial agents worldwide has led to a near ‘post-antibiotic era’ due to the epidemic of antibiotic tolerance and resistant microbes. A World Health Organization (WHO) report on antimicrobial resistance highlights the gravity of the current epidemic as well as placing doubts on our future ability to treat common clinical infections [1]. The report also alludes to the increasing urgency in discovering novel antimicrobial therapeutic agents and developing fresh strategies in the fight against antimicrobial tolerance.

We have previously reported the mechanism, safety and clinical applications (current and potential) for reactive oxygen species (ROS) therapy delivered as part of a symposium in Birmingham, UK in April 2016 [2]. ROS is the first entirely novel antimicrobial agent to reach early clinical use for several decades [3,4]. At present, ROS is available for clinical use in the form of Surgihoney Reactive Oxygen (SHRO), a natural honey with enhanced ROS activity, although there is a non-honey-based ROS gel due to be available for clinical use shortly. The clinical applications of ROS are topical, although ROS has also been used locally on internal structures and has the potential for other delivery mechanisms.

ROS particularly lends itself to conditions of soft tissue and epithelial surfaces (e.g. respiratory, urinary) where there may be a heavy bacterial bioburden with biofilm and chronic inflammation. Bacterial biofilms are a significant problem in clinical settings

by virtue of their increased tolerance towards conventionally prescribed antibiotics and their propensity to develop further antibacterial resistance. There is therefore a pressing need for the development of alternative therapeutic strategies that can improve antibiotic efficacy towards biofilms, thereby limiting antibiotic use and reducing the development of further resistance.

This review reports the output of a national symposium in the UK that looked at the effect of ROS on microbial biofilms. This could have significant therapeutic implications because antibiotics are typically poorly effective in biofilm-associated conditions. ROS delivered through engineered honey, as a topical gel or through other delivery mechanisms (as described below) could provide effective therapy in a wide range of biofilm-associated conditions.

2. Surgihoney Reactive Oxygen (SHRO) in wound biofilms

SHRO has been evaluated in chronic wounds in an open-label, multicentre study and has been shown, through its ROS activity, to reduce bacterial bioburden and biofilm, to support healing [5] and to prevent surgical site infections [6]. A recent study investigated the ability of SHRO and ROS prototypes with increased antimicrobial activity (SH2 and SH3) to prevent biofilm formation in vitro by 16 bacterial isolates [7]. For completeness, SHRO, SH2 and SH3 were compared with regularly used medical-grade honeys, including Activon manuka honey and Medihoney® manuka honey, as well as five antimicrobial dressings (AMDs). All of the honey products were serially double diluted in water from 1:3 down to 1:6144, and the lowest dilution achieving a statistically significant reduction in biomass of ≥50% compared with untreated controls was recorded. Although all of the honey products

were antibacterial and were able to prevent the formation of biofilms, SHRO was the most potent, with efficacy at lower dilutions than the other medical honeys. In addition, SHRO was superior in antibacterial potency to three commercially available AMDs that contain honey. It was concluded that SHRO is effective at preventing biofilms from forming and is superior to medical honeys and AMDs in these in vitro tests [7].

This in vitro study lends weight to the findings of clinical studies. It is difficult to demonstrate biofilms in vivo, and the pathological role of bacterial biofilms in chronic soft tissue lesions is debated. However, chronic soft tissue lesions can become heavily colonised with bacteria and are difficult to treat since they respond poorly to conventional antibiotic treatment. This leads to poor healing and potential transmission of multidrug-resistant (MDR) bacteria. ROS therapy appears to be highly active against biofilms in vivo, clinically effective, and can spare conventional antibiotic use and support infection control [5–9].

3. Reactive oxygen species as a novel adjunctive biofilm-targeted therapy in chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is a common upper airway infectious condition affecting up to 11% of the European population [10] and is a prominent risk factor for asthma. It is the second most common chronic disease in the UK (after arthritis), occurring more frequently than heart disease or high blood pressure. Afflicted patients often require life-long medical treatments and surgery to re-open and ventilate the sinuses, facilitating improved medication delivery. It is estimated that these medical and

surgical treatments cost the UK National Health Service in the region of £100 million per annum, placing a significant pressure on already overstretched resources. The impact of this disease is often underappreciated, with sufferers having a significantly impaired quality of life. CRS remains a difficult condition to control medically, with a significant number of patients requiring endoscopic sinus surgery [11]. It is well established that biofilm-positive CRS patients are likely to have increased infective exacerbations and slower wound healing and mucosal restoration rates, in addition to a higher level of antibiotic dependence [11]. *Staphylococcus aureus* biofilms in particular have been identified as one of the main causative agents for chronicity and recalcitrance of the disease [11–13].

The main aim of the project reported in this section was to investigate whether SHRO can be developed and repurposed for use as a novel biofilm-targeted and antibiotic-sparing therapy in CRS patients [14]. To evaluate this, in vitro biofilms formed by strains of methicillin-sensitive *S. aureus* (MSSA) isolated from CRS patients undergoing surgery were treated with SHRO for 24 h. Comparative analysis with untreated MSSA biofilms revealed that SHRO treatment caused a 2–3 log-fold reduction in the number of viable cells present in the biofilms (Fig. 1).

These preliminary data suggest that SHRO represents a viable antimicrobial adjunctive therapy in *S. aureus* biofilm-associated CRS disease. In view of the current epidemic of antimicrobial resistance, this new treatment has the potential to reduce antibiotic use and to improve outcomes after endoscopic sinus surgery.

4. Antibiofilm activity of Surgihoney Reactive Oxygen (SHRO) against non-typeable *Haemophilus influenzae*

Otitis media, one of the most common infections in young children, is primarily caused by the opportunistic pathogens *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (NTHi). These infections are the principal reason for repeated physician visits, contribute towards a significant socioeconomic burden, and most importantly represent the primary reason for antibiotic prescription in young children [15–17]. Recent research has determined that biofilm formation by pneumococcus and NTHi plays an important role both in colonisation and disease. Subsequent investigations have since focused on developing a better understanding of how these biofilms enable survival in the nasopharyngeal niche. This has led to the identification of a subset of proteins that are differentially expressed when pneumococcus undergoes the transition from a planktonic phenotype to the formation of a biofilm [18]. Of particular interest is the >2-fold increase in expression of pyruvate oxidase (SpxB), an enzyme responsible for the production of extracellular hydrogen peroxide (H_2O_2). Production of this ROS confers pneumococcus a competitive advantage over other pathogens that share the same nasopharyngeal niche, including NTHi [19]. It is possible that through exploitation of this susceptibility towards H_2O_2 that the tolerance of NTHi biofilms to antibiotic treatment could be diminished, or may yet provide an avenue for the development of an alternative therapeutic strategy that dispenses with the need for antibiotic prescription. SHRO that generates low concentrations of H_2O_2 over a sustained period of time represents such a product. Preliminary studies have shown that treatment of in vitro NTHi biofilms with low concentrations of SHRO has proven more

effective than the conventional antibiotic amoxicillin/clavulanic acid, highlighting its potential as a new strategy for targeting NTHI biofilm-associated infections.

5. Fungicidal effect of Surgihoney Reactive Oxygen (SHRO) on *Fusarium* biofilms

Chronic infections such as chronic wounds comprise 60–80% of infectious diseases in humans [20]. Colonisation of fungi in wounds is associated with the use of broad-spectrum antibiotics [21]. In patients with cutaneous trauma, *Fusarium* spp. may invade or colonise the burn wound [22]. Formation of biofilms contributes to the severity and delayed healing of chronic wounds [23–25]. Moreover, it has been shown that in 45 of 915 samples, *Fusarium* spp. formed biofilms in chronic wound infections [26].

Biofilms within wound infections have been linked to the pathogenesis of wounds and associated with delayed wound healing [23–25]. Recently, it was reported that in a total of 208 of 915 samples, fungi were identified from wounds within a 4-month study period. One of the most abundant moulds was *Fusarium* spp. [22]. It was also reported that as a result of low metabolic activities of biofilms, micro-organisms in biofilm forms are more difficult to eliminate with conventional antimicrobial agents than planktonic forms [26].

To remove or inhibit the growth of biofilms within wounds, looking for an ideal and novel method and/or agent that is non-toxic, inexpensive, practical and with less side effects than antimicrobial agents has been an active area of research. SHRO is an

agent that meets all of these criteria [14]. Given recent confirmation of the presence of *Fusarium* biofilms in wounds and their role in delayed wound healing, we designed a study to examine the in vitro effectiveness of different SHRO concentrations as well as the antifungals amphotericin B and natamycin against *Fusarium* planktonic and biofilm forms of growth using an XTT-based metabolic assay. In addition, the fungal cell wall biomarkers galactomannan (GM) and β-1-3-D-glucan (BDG) were measured to identify the effect of SHRO on the components of fungal cell wall. The results revealed that SHRO at a concentration of 50% markedly reduced biofilm formation in both isolates after 24 h of exposure. After 48 h of treatment, SHRO was able to prevent biofilm development of *Fusarium solani* and *Fusarium oxysporum* at concentrations of 25% and 100%, respectively. High levels of GM and BDG were detected after exposure of the biofilms to SHRO, suggesting that after exposure to SHRO the *Fusarium* biofilms were disrupted and GM was released. In a previous study, release of GM after exposure of *Aspergillus* spp. (*Aspergillus fumigatus* and *Aspergillus terreus*) to fluconazole, amphotericin B, liposomal amphotericin B and itraconazole was investigated [27]. The results showed that both formulations of amphotericin B and itraconazole reduced the GM level at the lowest doses tested. However, high doses of fluconazole had negligible effect on GM release, but at a concentration of 128 mg/L fluconazole increased the GM level [27]. These differences may result from several mechanisms of action. In a similar way, BDG was released at high levels (>500 pg/mL) in our study, which was the upper limit of the assay.

Different concentrations of SHRO reduced biofilm formation by both *Fusarium* spp. compared with control biofilms. This effect may largely depend on the density of

biofilm formation. SHRO at a concentration of 50% was found to disrupt established *Fusarium* biofilm after 24 h. Amphotericin B and natamycin also show a significant reduction in *Fusarium* biofilm at concentrations of 2 mg/L and 4 mg/L respectively. In contrast, for the planktonic form, concentrations of SHRO ranging from 25% to 50% had an effective minimum inhibitory concentration (MIC) against *Fusarium* planktonic cells. Both isolates were more susceptible to amphotericin B (1–2 mg/L) than natamycin (2–4 mg/L). The specific mechanism for releasing high GM and BDG after SHRO exposure cannot be explained without additional investigation. This is the first study reporting results of the effect of SHRO on the development of *Fusarium* biofilms as well as planktonic growth forms. It is also the first study examining the release of fungal biomarkers (GM and BDG) by *Fusarium* biofilms after exposure to SHRO and antifungal agents.

6. Innovative reactive oxygen species delivery systems

Currently, ROS is only available for clinical use as SHRO packaged in a sachet owing to its high viscosity. An ROS gel with the same efficacy, not using honey as a delivery mechanism, will be available shortly. The viscosity of SHRO can make it difficult to handle and to administer a controlled dose. Development of other physical formulations, which facilitate an application-specific release profile of ROS, is of great interest and could have wide clinical applications. It is conceivable that a carrier mechanism could be developed to deliver ROS at remote clinical sites.

When developing such a system, it is important to consider the underlying mechanisms of action. In the case of SHRO, addition of external water activates the production of hydrogen peroxide and reactive oxygen (Eq. 1). As a consequence, a

non-aqueous vehicle is required to avoid premature production of ROS before clinical application. Formulation of a non-aqueous product, in an appropriate physical form, would enable it to be stored and activated in situ. Owing to the wide range of bacterial species that SHRO is active against, there is a wide array of clinical applications in which it could be used. To date, much of the clinical use of SHRO has been topical. Therefore, initially this work has been focused on re-engineering what is currently in a sachet to a nebulisable spray, improving the ease of use and allowing for tailored release by altering the underlying formulation.

$$C_6H_{12}O_6 + H_2O + O_2 \xrightarrow{GOx} C_6H_{12}O_7 + H_2O_2$$

Eq. 1. Oxidation of glucose by glucose oxidase to produce gluconic acid and hydrogen peroxide [28].

An emulsion can be defined as a dispersion of droplets of one liquid in another in which it is not soluble or miscible [29]. Emulsions come in two basic forms, oil in water and water in oil. Every-day examples of emulsions include salad dressing, paint and cosmetics. To create an emulsion that is stable over time, surfactants, otherwise known as emulsifiers, are often used [30]. Surfactants are molecules that exhibit a hydrophilic head and a hydrophobic tail, which enables them to reduce the surface tension between phases in an emulsion, providing stability. In a solution containing a water phase and an oil phase, they orientate themselves with the head of the surfactant in the water phase and with the tail in the oil phase. This creates droplets of one liquid within another; the formation of water droplets within an oil phase is referred to as a reverse micelle (Fig. 2).

During the formulation of an emulsion, a shear force is typically applied to the continuous phase before addition of the dispersed phase. This allows for the generation of small droplets of the dispersed phase. Using a cup and vane set-up on an AR-G2 rheometer (TA Instruments, UK), the influence of temperature and shear rate on the efficacy of SHRO was established prior to emulsion formulation. The use of this geometry allowed for homogeneous control of temperature and shear rate.

A range of oils and surfactants were investigated with the aim of achieving a stable non-aqueous emulsion in which SHRO was encapsulated within reverse micelles. Promisingly, these initial studies demonstrated that it was possible to produce a stable formulation when SHRO was encapsulated by reverse micelles in paraffin oil (Fig. 2). This was achieved by using polyglycerol polyricinoleate (PGPR) as a surfactant. Importantly, the emulsion maintained its capacity to generate ROS when stored under ambient conditions (20 °C) for up to 4 weeks. Size analysis of the reverse micelles using optical microscopy revealed an average of 180 μm, however this was observed to vary significantly from 65 μm to 400 μm throughout the samples (Fig. 3). It is suggested that the size heterogeneity may have occurred as a result of coalescence of the micelles; this is where two or more particles may combine due to interface instability. This process may continue over time and is irreversible [31]. To address this, future studies will investigate producing a smaller and narrower distribution of particles, which ultimately will improve longevity and delivery.

Fundamentally, the rheology of this initial promising formulation may be modified to match that of a cream or spray; both of these physical forms would improve the ease

of application clinically. To demonstrate this potential, the emulsion was loaded into a pump spray bottle and manually nebulised (Fig. 4). This spray was directed into a beaker and was tested for the presence of peroxides both before and after the addition of water using Quantofix® peroxide test sticks (Sigma-Aldrich, UK). The test showed that before addition of water, peroxides were not produced and after addition peroxides were detected, as demonstrated by the blue colour of test stick ii in Fig. 4.

The outputs of this preliminary study demonstrate the ability to exploit formulation engineering to develop innovative ROS products that may be used as alternatives to current antibiotic-based treatments. Such research has the potential to address a number of unmet clinical needs. Notably, these innovations are timely to aid in the fight against antimicrobial resistance.

7. Conclusions

An earlier review reported on this novel ROS technology, the mechanism of action and potential therapeutic applications [1]. This review has examined in more detail the effects of ROS therapy on microbial biofilms. Biofilms in association with heavy microbial bioburden cause persistent infection in many clinical conditions. Most of these infections result from initial colonisation and have a connection to the exterior: wounds, burns, inflamed respiratory tract or bladder mucosa. Bioburden and biofilm cause significant pathology in these conditions, and conventional antibiotics are poorly active against biofilm-associated infection. Indeed, antibiotics in these conditions tend to result in greater antimicrobial resistance through selection pressure.

ROS has been successful in treating chronic wounds [4] and clearing MDR organisms, including methicillin-resistant *S. aureus* (MRSA), and carbapenemase-producing isolates from wounds and vascular line sites [7,8]. The significant antifungal activity of ROS on planktonic and biofilm-based microbes is documented in Section 5. Nebulised ROS has been evaluated in limited subjects to assess reductions in bioburden in chronically colonised respiratory tracts [14]. The work outlined in Sections 3 and 4 on the effects of SHRO on respiratory tract mucosa could have great implications for the treatment of a variety of persistent respiratory conditions. These are just the sort of conditions where conventional antibiotics are overused with limited clinical benefit and where ROS could play an important role in control of bioburden and biofilm. ROS technology could help patients with chronic colonisation and infection of the bladder with MDR bacteria.

Finally, research into ROS delivery formats is important to enable the delivery of active ROS to remote sites of infection while retaining antimicrobial activity. This symposium may prove to have had historic implications by presenting the first entirely novel antimicrobial technology for several decades and a technology with wide clinical applications that will provide one solution to help resolve the global crisis of infections caused by MDR microbes.

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Competing interests: MSD and JC have invested time and funds in the development of ROS technology. All other authors declare no competing interests.

Ethical approval: Not required.

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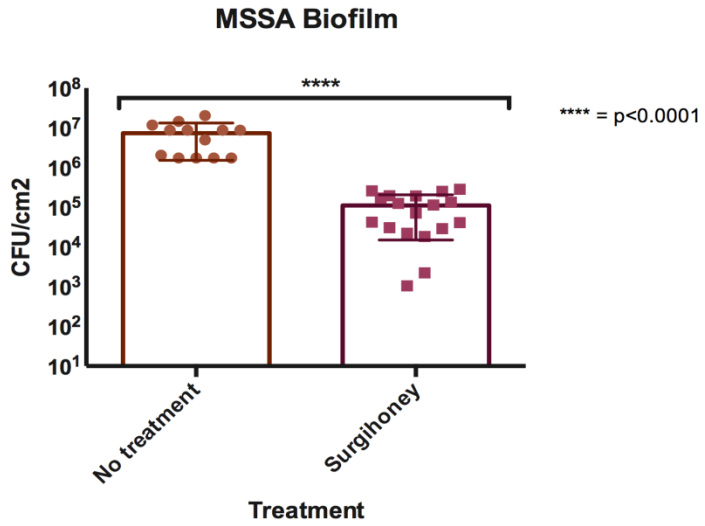

Fig. 1. Surgihoney Reactive Oxygen (SHRO) treatment reduces the viability of methicillin-sensitive *Staphylococcus aureus* (MSSA) biofilms. In vitro 48-h-old biofilms formed by MSSA isolates from chronic rhinosinusitis patients were treated with SHRO for 24 h and viability was measured by enumeration of CFU. A significant reduction in viability (2–3 log-fold) was observed following treatment with SHRO.

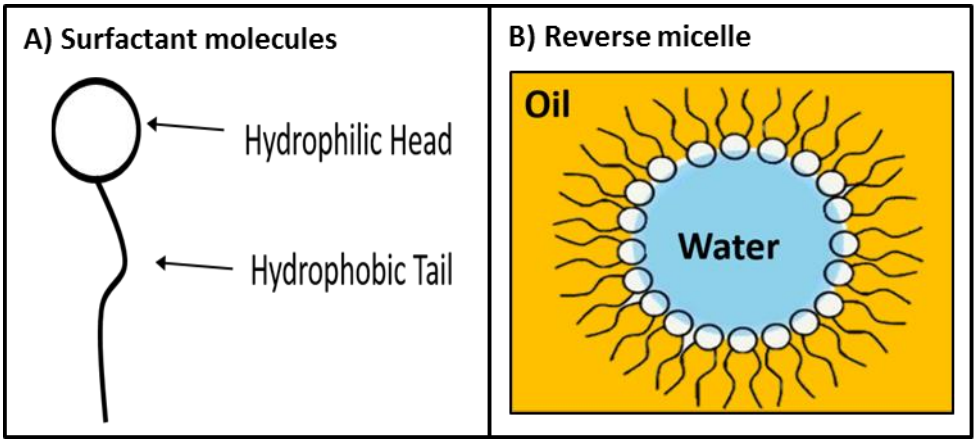

Fig. 2. Schematic illustrating (A) a surfactant molecule and (B) how they arrange to form a reverse micelle.

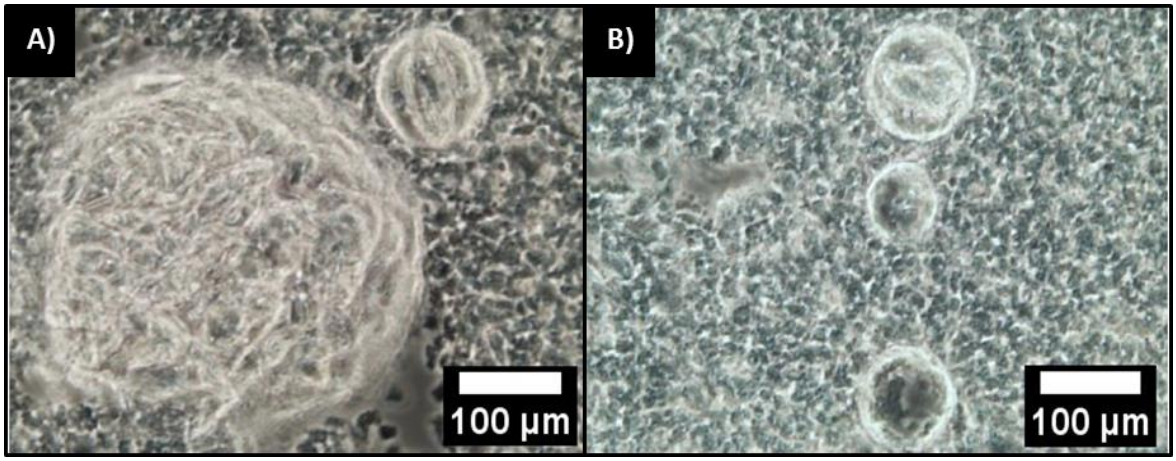

Fig. 3. Micrographs of successful reverse micelles containing Surgihoney™RO® in a continuous phase of paraffin oil demonstrating the presence of (A) coalesced droplets and (B) smaller stable individual droplets.

Fig. 4. (A) Surgihoney™RO® spray and (B) hydrogen peroxide test strips demonstrating no detectable concentration of peroxides before water addition (i) and production of ca. 3–10 ppm of detectable peroxides after water addition (ii).