A Delicate Balance:

Modulation of Reactive Oxygen Species after Sinus Surgery to Improve the Healing Process

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Thesis declaration

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Dr. Michael Gouzos

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Publications Arising During This Thesis

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- Gouzos, M., Ramezanpour, M., Bassiouni, A., Psaltis, A. J., Wormald, P. J., Vreugde, S., 2020, Antibiotics Affect ROS Production and Fibroblast Migration in an In-vitro Model of Sinonasal Wound Healing, Front Cell Infect Microbiol, 10, p110
- Gouzos, M., Cooksley, C., Feizi, S., Coenye, T., Psaltis, A.J., Wormald, P.J., Vreugde, S., 2020, *In Vitro* Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Treating *Staphylococcus aureus* Biofilms, prepared for submission
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- A Pro-Healing Antioxidant: Safety and Efficacy of Mitochondrially-Targeted Antioxidant Mitoquinone for Reducing Infections and Preventing Adhesions after Sinus Surgery, The Queen Elizabeth Hospital Research Expo, October, Adelaide, Australia
- *In Vitro* Safety and Efficacy of Mitochondrially-Targeted Antioxidant Mitoquinone after FESS, American Rhinological Society 66th Annual Meeting, Boston, MA, USA (virtual)

2019

- Choice of Postoperative Antibiotics after FESS Contributes to Rates of Revision Surgery, Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting, March, Brisbane, Australia
- A Delicate Balance: Modulating Reactive Oxygen Species after Sinus Surgery to Augment the Healing Process, The Queen Elizabeth Hospital Research Expo, October, Adelaide, Australia

2018

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Important Abbreviations and Chemical Formulas

C. elegans:	*Caenorhabditis elegans*
CFU:	Colony forming unit(s)
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without (*sans*) nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
ETC:	Electron transport chain
ECM:	Extra-cellular matrix
(F)ESS:	(Functional) endoscopic sinus surgery
H_2O_2:	Hydrogen peroxide
HNEC:	Human nasal epithelial cells
INCS:	Intranasal corticosteroids
LDH:	Lactate dehydrogenase
MTA:	Mitochondrially-targeted antioxidant
NAC:	N-acteyl cysteine
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
S. aureus:	*Staphylococcus aureus*
Th2/1:	T-helper 1 cell/T-helper 2 cell
UQ:	Ubiquinone

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Aims and Thesis Summary

The purpose of this thesis is to examine the role of reactive oxygen species in wound healing, with respect to the paranasal sinuses and in the dual context of chronic inflammation and surgery. It goes on to explore emerging therapies that modulate reactive oxygen species and act as wound healing adjuncts.

The work describes a body of translational research, utilising both *in vitro* and *in vivo* experiments aimed at improving postoperative outcomes. Focus was given to the prevention of postoperative infections and sinonasal adhesions, as these are significant issues in the realm of ENT surgery.

The thesis itself is composed of six chapters, each dealing with a different aspect of reactive oxygen species, as well as various established and novel therapies that interact with them in the postoperative period.

Chapter 1 serves as an introductory chapter and briefly explores the core concepts that underpin the subsequent chapters. It is subdivided into three sections. The first deals with chronic rhinosinusitis diagnosis and management (including sinus surgery), the second describes the stages and mechanisms involved in wound healing and the third explains the role of reactive oxygen species in this process.

Chapter 2 is a peer-reviewed publication, examining the modulation of reactive oxygen species by antibiotics given peri and post-operatively in ENT surgery, and the implications this may have for wound healing quality.

Chapter 3 is a manuscript describing the suppression of *Staphylococcus aureus* biofilms from the upper respiratory tract using a pair of novel mitochondrially-targeted antioxidants (MTAs).

Chapter 4 is a manuscript investigating the direct effect of these same antioxidants on fibroblast migration into freshly wounded cell monolayers, and the utility this has for limiting scar formation.

Chapter 5 is a manuscript tying these concepts together using an *in vivo* murine model of infected cutaneous wound healing, to investigate whether these agents concurrently improve wound healing and suppress active staphylococcal biofilm infection.

Conclusions and reflections on future directions for this body of research are drawn in Chapter 6. Modulation of reactive oxygen species represents a promising treatment avenue for chronic rhinosinusitis, in unison with existing treatments such as sinus surgery. Mitochondrially-targeted antioxidants have the potential to utilise this mechanism to address multiple factors driving disease persistence at once, providing hope for an elusive definitive treatment.

Literature Review

1.1. Chronic rhinosinusitis

1.1.1 Definitions

Chronic rhinosinusitis (CRS) is part of a spectrum of inflammatory conditions involving the nasal cavity and the surrounding paranasal sinuses. Under normal circumstances, these are air-filled spaces within the skull that are lined with respiratory mucosa and communicate through a series of small apertures or ostia. There are usually four paired paranasal sinuses that develop within the skull, conventionally named for the bones in which they arise. [1, 2] The maxillary and ethmoid sinuses lie lateral to the nasal cavity, with the frontal and sphenoid sinuses abutting the midline. [2]

Most rhinology panels and expert guidelines agree on the common nomenclature of rhinosinusitis instead of sinusitis, to acknowledge that rhinitis and sinusitis usually co-exist and are part of the same fluid disease process. [3] The most contemporary consensus statements and positions papers on rhinosinusitis define the entity in adults as 'sinonasal inflammation persisting for more than 12 weeks', a definition that has remained largely consistent for the past two decades. [3, 4] Whilst the precise choice of a 12-week time period is somewhat arbitrary, this delineation between 'acute' and 'chronic' rhinosinusitis has been useful in establishing reproducible protocols for investigating and treating patients with these two very different disease entities.

Whilst rhinosinusitis may manifest from a number of endotypes, or as a variety of phenotypes, the essential diagnostic criteria are agreed to be [3, 4]:

- nasal obstruction/ congestion/ blockage
- mucopurulent nasal discharge that may drain anteriorly (often termed *rhinorrhoea*) or posteriorly (often termed *post-nasal drip*)

The following symptoms may also be present, however, importantly, they do not clinch the clinical diagnosis of CRS in isolation [3, 4]:

- facial pain/ pressure/ fullness
- decreased or loss of sense of smell (hyposmia or anosmia, respectively)

Symptoms alone have a high sensitivity but an unacceptably low specificity in diagnosis of CRS, [4] and so for this reason the diagnostic workup should also include objective findings on nasal endoscopy (purulence, polyps, or oedema) or mucosal changes evident on imaging of the sinuses, usually computed tomography (CT). [3]

1.1.2 Aetiology

Though a large body of literature exploring the aetiology of CRS exists, there is yet to be a clear consensus on its precise mechanism of development. Broadly speaking, it is born of an aberrant relationship between the host's immune system and their environment. A number of existing hypotheses explain some, but not all, of the observed disease processes and relationships that typify CRS. There are significant overlaps and interplay between theories grounded in **environmental** aetiologies and **host** aetiologies, with the cause and effect sometimes being vague or indistinguishable.

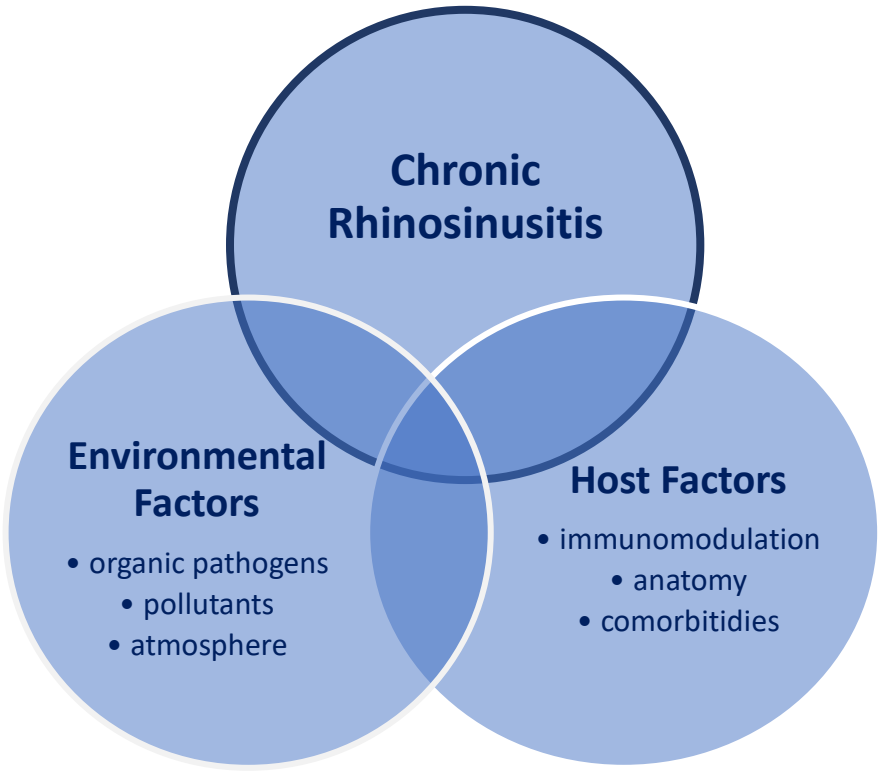


Figure 1.1 Venn diagram of CRS aetiology

Environmental Factors: Much of the early work done on the aetiology of CRS attempted to identify a single organic pathogen that may trigger the onset of the disease. Early studies postulated this initiating organism may be fungal, although this was soon debunked in favour of a more modulatory role in the CRS paradigm [5]. The presence of viruses has also been considered as a potential catalyst for CRS without nasal polyposis, which may portend an increase in disease severity. [6]

Bacteria, or more specifically dysbiosis of the sinonasal microbiome, has overtaken fungal elements as a more likely pathogenic driver of the disease. There is also a problematic lack of consensus in the literature regarding the bacterial constituents of a healthy sinus, particularly when a core microbiome comprised of *Corynebacterium, Staphylococcus, Streptococcus, Haemophilus* and *Moraxella* is known to exist in both healthy and CRS

cohorts. [7] The superantigens produced by bacteria have also been proposed as possible downstream instigators of the CRS disease process, as it has been shown that these exotoxins drive the inflammatory process and the development of nasal polyposis by stimulating a range of lymphocyte and myeloid intermediates. [8, 9] However, these superantigens do not always result in an inflammatory response when they are present, meaning they may also be just an incidental 'bystander'. [10]

More recently, the concept of biofilm and small colony variant (SCV) lifestyles have garnered significant attention, as mechanisms by which micro-organisms may gain a foothold in the sinuses and drive a chronic inflammatory response [11-13] Biofilms in particular present an urgent therapeutic challenge, as biofilm-forming bacteria have been shown to increase resistance to antibiotics by as much as 1000-fold, as well as providing an evasive mechanism against the host immune response. [14]

Bacteria in the sinuses begin their transition from planktonic organisms to a biofilm lifestyle by adhering to the host mucosa and forming a distinct microcolony. Once a predetermined 'quorum' of bacteria is reached at this attachment site, they will begin to secrete a complex of polysaccharides, proteins and nucleic acids- forming a protective matrix around themselves. [15, 16] As this 'biofilm' develops, some of these bacteria begin to differentiate into a distinct biofilm subgroup, with a less metabolically active profile designed to regulate expansion of the matrix and provoke less of a response from the host. Meanwhile, free-floating planktonic organisms continue to be released from the edges of the biofilm, forming new microcolonies within the sinuses and perpetuating further biofilm formation. [12, 13, 17]

Different biofilm species are associated with different disease phenotypes, particularly in the upper respiratory tract. *Haemophilus influenzae* biofilms are typically found in patients with milder disease, whereas *S. aureus* is associated with a more severe, recalcitrant pattern [18]. *S. aureus* is a particularly problematic pathogen because it also has the potential to cause intracellular infections, which can evade host immunity in a similar manner to biofilms. [12, 13]

Finally, it has been suggested that diseases that can cause disruption of the mucosal barrier and ciliary function may lead to increased colonization of pathogenic micro-organisms, with a corresponding upregulation of the adaptive immune system and its inflammatory response. This concept manifests in several disease processes with which CRS is well-known to have a high incidence, such as cystic fibrosis and primary ciliary dyskinesia. [19, 20]

Host Factors: The other half of the CRS equation describes host factors that may potentiate or 'multiply' disease development.

Allergy, as a prime example, has long been associated conceptually with rhinitis, although its role in the sinuses is more controversial. It is certainly plausible that allergenic swelling of the nasal mucosa leads to stasis of mucus in the paranasal sinuses and subsequent infection, though the causal relationship remains unclear. [3]

In 1968, Samter and Beers first described an association between non-steroidal anti-inflammatory drug intolerance and the presence of both asthma and CRS with polyposis. Known as Samter's triad, these combined comorbidities portend a more severe form of CRS, which is reflected by worse radiological disease scores and surgically recalcitrant

disease. [21] A review the following decade of over 6000 patients with asthma found that 7% of had clinical evidence of nasal polyps [22] and in a more recent study, 36-96% of patients with aspirin sensitivity were shown to have evidence of CRS with polyposis. [3] Immunocompromise has also been shown to be a predictive factor in CRS development, with one prospective study of 74 HIV positive patients showing a CRS incidence of 34%. [23]

As there is no unifying mechanism present in any of these theories that encapsulates all cases of CRS, the disease has been accepted, for the time being, as having a multifactorial aetiology drawing from host and environmental factors.

1.1.3 Epidemiology and disease burden

Prevalence estimates for CRS are limited by the quality of data in the literature, which often employ self-reported, subjective symptoms as a measure of severity, rather than the formal diagnostic criteria discussed in chapter 1.1.1. [3, 4] For example, one publication using self-reported 'sinus trouble' for greater than 3 months in the year, estimated the prevalence of CRS at 15.5% of the total population in the United States, [24] whilst a Canadian study asking patients if the they had been diagnosed with CRS by a health care professional found its presence in 5.7% of females and 3.4% of males. [25] Even lower rates of CRS have been described in a Korean study which found symptoms of rhinosinusitis in only 1% of the population. [26] A late 2019 Australian study by Harvey and colleagues [27] found that, compared to other chronic diseases in Australia, CRS prevalence sits at approximately 9.8%, making it more common than anxiety (9.6%), depression (8.9%), diabetes mellitus (5.1%) and ischaemic heart disease (3.3%).

Admittedly, some of this difference may also be reflective of straightforward geographic and demographic variability. For example, the US Centre for Disease Control's National Health Interview Survey consistently finds variations in sinusitis incidence between regions (e.g. 9.8% in Northeast vs. 13.8% in Southern US, 2017) and race (12.7% 'White' vs 10.7% 'Black' vs. 8% 'Hispanic', 2017). [28] Taken together, these studies also highlight the problems inherent in quantifying this disease using self-reported data, as opposed to objective data recorded by a specialist medical practitioner. Whilst less costly and time-consuming, they clearly have a potential for large discrepancies in results, likely due to reporting and recall bias.

In any event, there is enough evidence in the literature to confidently conclude that CRS is a substantial problem affecting millions of people worldwide.

1.1.4 Medical therapy

Prior to considering any surgical intervention, the accepted paradigm of managing patients with CRS is to begin by trialling 'maximal medical therapy'. [29] Management algorithms are traditionally based on the most prominent phenotypes of the disease; CRS with polyposis, which is T-helper 2 (Th2) related and CRS without polyposis, more often not Th2 related. [30] To aid in this delineation, there is evidence that blood eosinophils are a reasonable biomarker to predict eosinophilic CRS with or without nasal polyposis. [31]

Therapy is variable between institutions and lacking true consensus, but usually comprises topical or systemic corticosteroids, saline douches and possibly a course of oral antibiotics. [32] More recently, Th2-targeting biologics such as anti-IgE, anti-IL4Rα, anti-IL5 and anti-IL5Rα have also garnered interest as potential additions to this

armamentarium for patients with CRS with polyposis, but are still in their infancy and are not yet widely available to prescribe. [33]

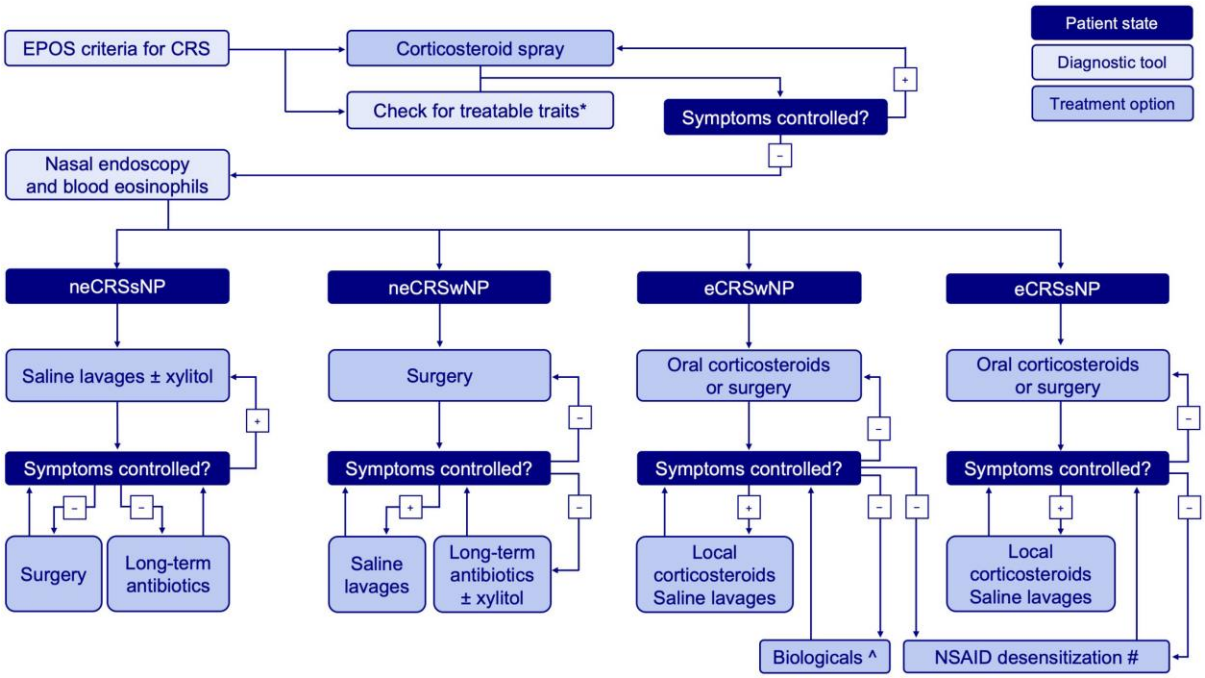


Figure 1.2 Chronic rhinosinusitis treatment algorithm. From Fokkens and Reitsma, 2019 [33]

EPOS: European position paper on rhinosinusitis and nasal polyps; CRS: chronic rhinosinusitis; neCRS: (low/non) eosinophilic CRS; sNP: without nasal polyps; wNP: with nasal polyps; NSAID: nonsteroidal anti-inflammatory drugs.

*Treatable traits: smoking, allergy, occupation and mucociliary clearance deficits.

^Biologicals can be considered but additional criteria for this exist in most countries.

#NSAID desensitization should only be considered after a positive provocation in patients, without contraindications for long-term use of high doses of aspirin

If the patient's disease proves recalcitrant to medical treatment, or if the side effects prove intolerable or unacceptable, their otolaryngologist may then recommend sinus surgery to address the disease and/or enhance further medical management. [34] This

will ideally lead to a period of relative improvement in symptoms, after which the disease will either recur or stay senescent.

Disease recurrence will often portend further sinus surgery, especially if it is relatively rapid. Several factors such as surgeon experience, patient compliance, and disease biology will ultimately influence this outcome, and these can be difficult to delineate. [35] Reported rates of revision surgery for chronic rhinosinusitis following FESS range between 0% and 50% depending on length of follow up, with a recent study finding a 4% overall revision rate at 28 months over 424 CRS patients using a single rhinologist. [35]

Antibiotics: Antibiotics are used in CRS both for their antibacterial and immunomodulatory properties. Short duration antibiotics (< 4 weeks) can be prescribed in the setting of acute exacerbations of CRS and should be directed by culture sensitivities. [36] Some centres advocate antibiotics as part of 'maximal medical therapy' even in the absence of purulent discharge. [32] A Cochrane review in 2016 [37] found very little evidence that systemic antibiotics are effective in patients with chronic rhinosinusitis.

In certain circumstances, antibiotics are instead prescribed on a long-term basis (>4 weeks). Macrolides are the most favoured antibiotic for this style of prescribing, as a result of a published record of success treating other pathologies of the airway. For example, long-term, low-dose erythromycin treatment changed the 10-year survival rate of diffuse panbronchiolitis from 25% to over 90 %, and was shown to simultaneously clear concurrent CRS. [38, 39] In cystic fibrosis, a large body of randomised controlled trials have shown a beneficiary effect using the macrolides clarithromycin and azithromycin. There are undisputed effects on inflammatory markers, such as IL-8, IL-4, interferon-gamma and TNF-α as well as reducing the rate of exacerbations and reducing decline in

lung function. [40] Although not all studies have shown an overall improvement in quality of life, it is now a recommended adjunctive treatment in cystic fibrosis. This effect is seen at lower doses than those used to treat infection, and in point of fact, it occurs despite the absence of common pathogens or in the presence of non-sensitive pathogens. Combined with the well documented anti-inflammatory effects of macrolides *in vitro* it has led to the concept of macrolides acting in an immune-modulatory, rather than anti-bacterial fashion.

In the setting of CRSsNP, Wallwork and Cervin et al. performed a landmark Australian RCT investigating the benefit of macrolides. These patients received roxithromycin for a period of 3 months and demonstrated significant improvements in quality of life, nasal endoscopy, saccharine transit time, and IL-8 levels in lavage fluid. Improved outcome measures were particularly noted in patients with low serum IgE levels. [41] Another RCT using the alternate macrolide azithromycin for 3 months failed to demonstrate any significant improvement over placebo. [42] Yet another study compared 12 weeks of erythromycin directly with FESS. Both treatment modalities improved all symptoms significantly, except for nasal volume, which was better in the surgery group. [43]

The benefit of other antibiotic classes has also been investigated in CRSwNP patients. Van Zele et al. investigated the tetracycline doxycycline in this population due to the theory that *S. aureus* toxins may be important disease modifiers in nasal polyposis. [44] They found that doxycycline reduced the size of nasal polyps and systemic markers of inflammation. [45]

For now, there is an agreement amongst experts that long-term antibiotic treatment should be reserved for patients where nasal corticosteroids and saline irrigation has failed

to reduce symptoms to an acceptable level. [3, 4] Data suggests that the population with high serum IgE are less likely to respond to macrolide treatment and the ones with normal IgE more likely to do so. [46] Other choices, such as long-term treatment with doxycycline, could turn out to be promising alternatives but further studies are warranted.

A concern with long-term antibacterial treatment is the emergence of resistant bacterial strains, especially when using a dose below the minimal inhibitory concentration. In a placebo randomised, double-blind trial studying the effect of exposure of oral streptococcal flora of healthy volunteers to azithromycin and clarithromycin, there was definitive proof that antibiotic use was an extremely important driver of antibiotic-resistance. They concluded that physicians prescribing antibiotics in the upper aerodigestive tract should take into account this important ecological side-effect of antibiotics. [47] One also must bear in mind the potentially harmful interactions long-term antibiotics may have with a patient's other medications, such as anticoagulants, antiepileptics, immunomodulators and antidepressants. Nasal swabs with microscopy, cultures and sensitivities every 3 months during a long-term antibiotic regime is advisable, to monitor the risk of the development of resistant bacterial strains. [3, 4] Other well-known side effects of antibiotics include gastrointestinal upset, skin rash and reversible elevation of liver enzymes.

Topical: One of the ways in which rhinologists have attempted to maximise antibiotic therapy whilst also circumventing the unwanted side-effects of antimicrobials is with topical therapy. This modality has the major advantage of delivering antimicrobials at a much higher dose than would be possible systemically, as the exposure is superficial and

much of the drug is not retained by the host. A number of different topical solutions have been used with different treatment periods.

One placebo-controlled RCT evaluated the efficacy of mupirocin rinses for the treatment of recalcitrant CRS with *S.aureus*. Whilst this treatment eradicated *S.aureus* from the nasal cavity in the majority of individuals and resulted in improvement in endoscopic scores, there was no significant change in the quality of life of these patients compared to those receiving placebo. [48] Not all studies mention side effects, but the most common seem to be intra-nasal stinging, burning sensation, moderate pain, throat irritation, cough and dry skin. [3] Given that the majority of these patients will also go on to recolonize *S. aureus* within months [49] one must question the long-term utility of such treatments. In the short-term however, it is possible that this microbiome 'reset' may be of benefit to certain subgroups.

Corticosteroids: Corticosteroids are an important component of medical management for CRS patients and can be delivered either topically or orally. These medications affect eosinophil function by directly reducing eosinophil viability and activation [50-52] or by indirectly reducing the secretion of chemotactic cytokines by nasal mucosa and nasal polyp epithelial cells. [53-55] A recent review and meta-analysis of topical intranasal corticosteroids (INCS) in CRSsNP patients found significantly improved symptom scores in the treatment group compared to those receiving placebo. [3] Large volume sinus squeeze bottle delivery methods like FLORinse ™ (Neilmed) bottles were also more effective than other nasal delivery methods, but there was no concomitant difference in endoscopic appearance, nor any difference between different steroid types. [3] Patients with CRSwNP

have also been shown to benefit from INCS; these patients demonstrate significant improvements in symptoms, polyp size, polyp recurrence rates and nasal airflow. [3]

Oral, systemic steroids may also be of benefit in CRSsNP patients, although high- level evidence is currently lacking. A recent systematic review of 30 studies on systemic steroid use in CRSsNP patients identified that the efficacy of single-modality oral steroid use in CRSsNP remained untested. [55] Two trials with a total of 78 participants met the inclusion criteria of a recent Cochrane review exploring the benefits of oral steroids in CRS patients. [56] They concluded there might be an improvement in symptom severity, polyps size and condition of the sinuses when assessed using CT scans in patients taking oral corticosteroids when these are used as an adjunct therapy to antibiotics or intranasal corticosteroids, but the quality of the evidence supporting this is low or very low. It is unclear whether the benefits of oral corticosteroids as an adjunct therapy are sustained beyond the short follow-up period reported (up to 30 days), as no longer-term data were available. There were no data in this review about the adverse effects associated with short courses of oral corticosteroids as an adjunct therapy.

The side effect profile of corticosteroids needs to be balanced against any benefits. Whilst long-term use of topical INCS is generally considered safe due their low bioavailability (<1%), [57] a number of patients still experience side effects related to their use which may include epistaxis, itching, sneezing and dry nose. [3] Oral steroids have the potential for more significant side effects, particularly when given at higher doses for longer durations. The data on the adverse effects associated with short courses of oral corticosteroids indicate that there may be an increase in insomnia and gastrointestinal

disturbances, but it is not clear whether there is an increase in mood disturbances. [56] More serious side effects from longer term use may include changes in bone mineral density, fat metabolism, proximal muscle strength, appetite and glucose tolerance. Patients are also at risk of early cataract formation and suppression of the pituitary-hypothalamic axis. [3] A frank discussion between the clinician and patient regarding the risks and benefits of steroid use should always be undertaken prior to their prescription.

Saline douches: Saline irrigations are another useful component of the pre- and post-operative care of patients with CRS. Irrigation of the sinonasal cavity can be limited pre-operatively by anatomical factors. In particular, the frontal recess and sphenoid sinuses are poorly penetrated in their native state. [58] Whilst not as beneficial as INCS, it is now generally accepted that saline irrigations are advantageous in the treatment of the symptoms of CRS when used as a sole treatment modality or adjunct. [59]

Combining these various types of treatment will allow many patients to avoid surgical intervention for the management of CRS. However, even if surgery is performed, these therapies continue to play an important role.

1.1.5 Surgical management

Functional Endoscopic Sinus Surgery: Functional endoscopic sinus surgery (FESS) is the term used to describe surgery to the paranasal sinuses that aims to preserve its self-regulatory capacity. FESS is built on the concept of restoring health in the diseased sinuses by re-opening the drainage pathways impacted by sinus pathology, thus improving ventilation and mucociliary clearance.

Originally described by Messerklinger and popularized by such proponents as Stammberger, [60] the technique has evolved over time and includes a spectrum of procedures ranging from middle meatal antrostomies with anterior ethmoidectomies ('mini' FESS), to complete fronto- spheno-ethmoidectomies ('Full House' FESS). Some patients with more extensive disease go on to receive a Draf-III [61] drillout procedure of the frontal sinus and/or extended surgery of the maxillary sinuses; usually canine fossa trephination (CFT) or mega-antrostomy. [62]

FESS has been shown to offer patients improvements in quality of life and numerous objective observational and radiological measures of CRS disease severity. [3, 4] The other equally important benefit of surgery is the improved delivery of topical treatments to the nasal cavity. [63]

Potential complications: In counselling a patient that may need to undergo FESS, the surgeon must not only consider the potential advantages but, perhaps more importantly, illustrate and appreciate its risks. These include major complications, which are rare and often immediate, and minor complications that occur more frequently.

Major complications of FESS include [64]:

- significant haemorrhage: intranasal, intraorbital or intracranial
- injury to a major blood vessel abutting the sinuses, such as the internal carotid artery (with the introduction of newer technologies and endoscopic techniques, this rate has dropped to 0.001%) [65-67]
- injury to orbital muscles ± resultant diplopia
- penetration into the skull base ± dural injury, CSF leak and, rarely, a resultant meningitis

Despite their severity, major complications in FESS are a very rare occurrence with an overall incidence rate of less than 0.01%, regardless of surgical approach utilised. [68]

Minor complications are still rare, but much more common than the major variety. These include postoperative epistaxis, damage to the lamina papyracea and adhesion formation. [67] Of these, adhesions or synechia are the most common occurrence with an incidence of 15-30%. Along with nasal stenosis, they can contribute to a difficult post-operative course for the patient, interfering with normal mucociliary transport and function, eventually leading to repeat obstruction of the sinuses and eventual requirement of revision surgery. [69-71] In fact, approximately 10-15% of patients that undergo sinus surgery will require revision surgery within five years, with adhesions making a significant contribution by making effective aftercare significantly more difficult. [35, 72]

Postoperative care: Medical management of CRS has been addressed elsewhere in this review. The guiding principles of both peri-operative and non-operative treatment for CRS have significant overlap despite the clear variation in context.

Perioperative antibiotics: The use of perioperative antibiotics in surgery gained traction in the 1960s, through the seminal work of Burke [73] who radically changed surgical practice by demonstrating in an animal model that antibiotics given just prior to incision prevented subcutaneous infection in fresh surgical wounds. This paved the way for clinical trials into perioperative antimicrobial prophylaxis in humans [74] and led in turn to a deluge of scientific evidence on their use. Clinical trials in antimicrobial prophylaxis have continued to elucidate many controversial issues across a number of surgical and procedural subspecialties: the value of prophylaxis in clean procedures [75], the most appropriate duration of its administration [76], multi-resistant organisms and the role of certain drugs such as aminoglycosides and vancomycin [77], treatment implementation [78, 79] and the value of oral vs. parenternal drug administration. [80] Overall, antimicrobial prophylaxis has achieved a solid scientific footing based on numerous prospective randomized trials, alongside robust evidence of cost-efficacy. [81]

The use of perioperative antibiotics has become widely adopted in rhinology, where procedures are often performed on an area of the body that is normally exposed to the environment, classifying it as clean-contaminated surgery [82]. Patients are commonly prescribed a 5 to 14-day course of a broad-spectrum oral antibiotic in the postoperative setting, with the aim of reducing rates of infection, crusting and nasal discharge, whilst concurrently optimising subjective sinonasal symptoms. Some otolaryngologists suggest prescribing antibiotics only if an infection is noticed at the time of surgery, targeted to the

intraoperative culture result [83], while others have asserted that they convey little to no benefit in a majority of cases. [82, 84]

Corticosteroids: Steroid therapy has been advocated to reduce excessive inflammation and maladaptive wound healing if given perioperatively. [85] This is particularly true if there is severe nasal polyposis, with systemic steroid being administered first in a tapered dose fashion , before switching to topical dosing 1-2 weeks postoperatively. [83, 85] A Cochrane review found low quality evidence that, after using oral corticosteroids for two to three weeks as an adjunct to surgery, there is an improvement in health-related quality of life and symptom severity in patients compared with placebo or no treatment. [86] However, this benefit was not seen at three to six months after the end of the oral steroid treatment period.

Saline rinses: Surgery to the sinuses has been shown to improve the penetration of nasal irrigations, with the critical sinus ostial dimension required to allow adequate penetration in 95% of cases identified at 3.95 mm. [87] Proposed advantages for postoperative saline irrigation include improved mucociliary clearance, reduced crusting, pus and debris, reducing oedema and removing the fibrinous exudate bridging the middle meatus. [83]

A Cochrane review examined the evidence for saline irrigation in patients with CRS, concluding that it increases the mucociliary clearance and appears to be useful for symptom management, whilst its effects in the postoperative period was less clear [88]. The group suggests that there is some benefit of daily, large-volume (150 ml) saline irrigation with a hypertonic solution when compared with placebo, but the quality of the evidence is low for three months and very low for six months of treatment.

Sinonasal debridement: Many rhinologists advocate an intensive debridement regime postoperatively, describing the advantages of debridement as a reduction of trapped mucus to re-infect sinuses, removal of nutrients for bacterial growth and removal of bony fragments that may propagate infection. [89]

Other centers and surgeons recommend minimal intervention in the postoperative period and describe equivalent results. In a review of 120 patients that underwent minimal debridement at 2 weeks postoperatively, one study reported no difference in adhesion outcomes compared to more aggressive care. [89] The paediatric population also provides interesting insight, as it is usually not possible to perform post-operative debridement in these patients. Even with tighter anatomy and no opportunity to debride, success rates in these patients are comparable to intensively debrided adult counterparts. [89, 90]

In this same vein, it is important to consider possible adverse effects on the wound healing processes that may occur due to debridement. One study found that debridement in the first week after ESS resulted in epithelial avulsion in 23% of cases, while debridement during the second week did not have this effect. Therefore, those authors recommended debridement be withheld until the second week. [89]1

Finally, a Cochrane review of this controversial topic was uncertain about the effects of postoperative sinonasal debridement, as it deemed there was a high risk of bias in the included studies and low-quality evidence. [91]. Low-quality evidence suggests that postoperative debridement is associated with a significantly lower risk of adhesions at three months follow-up. Whether this has any impact on longer-term outcomes is still unknown, and it may make little or no difference to disease-specific health-related quality of life or disease severity. [91]

While much has already been achieved in the realm of postoperative care following sinus surgery, it is clear that there is potential to further improve both subjective and objective outcomes for patients, while also reducing the risk of recurrent disease and revision sinus surgery. Novel treatments that counteract both the host and environmental drivers of CRS are needed to address this truly multifactorial disease.

1.2. Wound Healing

1.2.1 Normal physiology

Wound healing is a regenerative process that constitutes an effort by an organism to protect its tissue from repeated injury, prevent the loss of vital substances and to replace or repair damaged structures. [92] The subsequent wound healing outcome will lie on a continuum between complete replacement of injured tissue with regenerated cells or, less desirably, fibrous scar tissue formation. Many growth factors and cytokines are responsible for coordination of the processes involved, including inflammation, cell proliferation, matrix deposition and remodelling. These factors activate their target cells by binding to the corresponding high-affinity surface membrane receptors.

There are four well described, overlapping stages of wound healing: the coagulation phase, the inflammatory phase, the proliferative phase and the maturation/remodelling phase. [92, 93] Each phase features a distinct variety of cells and inflammatory mediators undertaking a synchronised set of processes to gradually heal the injured tissue. [94]

Coagulation: Trauma, be it surgical or organic, results in the obligatory rupture of vessels and exposure of sub-endothelial collagen to platelets. In the presence of thrombin and fibronectin, this contact between platelets and collagen results in the release of cytokines and growth factors from platelet alpha-granules, including platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), platelet-activating factor, fibronectin, and serotonin. [95] Fibrin within the clot also stimulates the release of PDGF, epidermal growth factor, insulin like growth factor-1 (IGF-1), TGF-β, and fibroblast growth factor (FGF). [93] This locally established fibrinous clot persists as an important scaffold for

migrating cells, including neutrophils, monocytes, fibroblasts, and endothelial cells. [96-98]

Inflammation: In the first 24-48 hrs after injury there is an increase in vascular permeability due to inflammation and the release of reactive oxygen species and prostaglandins. A building concentration gradient of chemotactic substances such as complement factors, interleukin-1, tumour necrosis factor-alpha, TGF-β, platelet factors and bacterial products drives this process further. [95] These locally released cytokines and growth factors result in the chemotaxis of polymorphonuclear neutrophils over the next 24-48 hours. These neutrophils use endogenous integrins and various enzymes to penetrate from the circulation and into the extracellular matrix.

After the first 72 hours these migrating neutrophils begin to be overtaken by monocytes, which then become activated by local cytokines and endotoxins. [92] These activated monocytes play a central role in propagating wound debridement, matrix synthesis and angiogenesis [99] through the secretion of growth factors. [93] If a prolonged inflammatory phase occurs, during a postoperative microbial infection for example, this inflammatory activity may go on to drive an excessive phase of fibroplasia. [95]

Proliferation: The proliferation phase lasts between 3-21 days and is characterised by a robust migration and mitosis of fibroblasts and epithelial or endothelial cells throughout the wound bed. Macrophages located in the nasal lamina propria provide continuing stimulation of the proliferation of these cells. [93] Cytokines from platelets and macrophages are responsible for the migration and attraction of fibroblast to the wound area. This phase is characterised by angioneogenesis and a corresponding increase in

blood flow to the healing tissue; easily recognised clinically through the development of pink granulation tissue.

Fibroblasts play an important role in wound healing because they are ubiquitously present in the connective tissue of every organ system, where they deposit and remodel extracellular matrix (ECM). [100] Interestingly, there is significant heterogeneity amongst fibroblasts derived from different tissue types, different stages of maturation and their activation status. [101] This heterogeneity contributes to substantial phenotypic differences in performance during wound healing, including ECM deposition and organization, secretion of growth factors and cytokines, and immunomodulation. [102]

Once fibroblasts have migrated within the wound they switch their major function to collagen synthesis, reaching their maximum production in the first 2 weeks, whilst overall wound collagen levels peak within 3 weeks. [92] Epithelial regeneration and migration proceeds inwards from the adjacent, uninjured areas and begins a few hours post injury, with an estimated velocity of 4 μm/hour within the nasal cavity. [103, 104] The epithelial cells at the wound edge slowly develop cytoplasmic extensions into the wound area.

Four different processes occur simultaneously to allow for complete re-epithelialisation: epithelial cell migration, multiplication, reorientation and differentiation from respiratory basal cells (the main source of epithelial cells). [105] Whilst epithelial regeneration occurs rapidly, polarisation, ciliation and differentiation can take as long as several months. [106, 107]

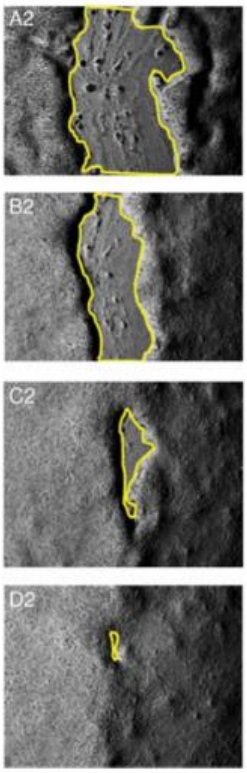


Figure 1.3 Nasal epithelium wound healing. From Wise et al, 2013 [108] 10x microscopic *in vitro* photographs of sinonasal epithelial monolayer wound healing; from initial wound (A2) to 12-hour (B2), 24-hour (C2) and 36-hour (D2) intervals, with wound edge outline overlays.

Remodelling: Nasal extracellular matrix remodelling, cell apoptosis and wound remodelling continues for approximately 6 months after surgery, [93] although a full thickness injury through mucosa to bone or cartilage may take much longer and not be completely mature for 18 months or more. [109] Ongoing inflammation can up-regulate and prolong the process of remodelling, and as this remodelling occurs the collagen types begin to be substituted for one another. In the initial stages of healing the ECM will be composed mainly of hyaluronic acid, fibronectin and collagen types I, III and V. As remodelling occurs collagen type III is replaced with collagen type I. [110] Most cells also produce proteinases that are able to degrade the ECM, including serine proteinases, cysteine proteinases and matrix metalloproteinases. [111] The 'tug-of-war' between collagen synthesis and lysis gradually results in an increase in the wound tensile strength and resilience over time. [93]

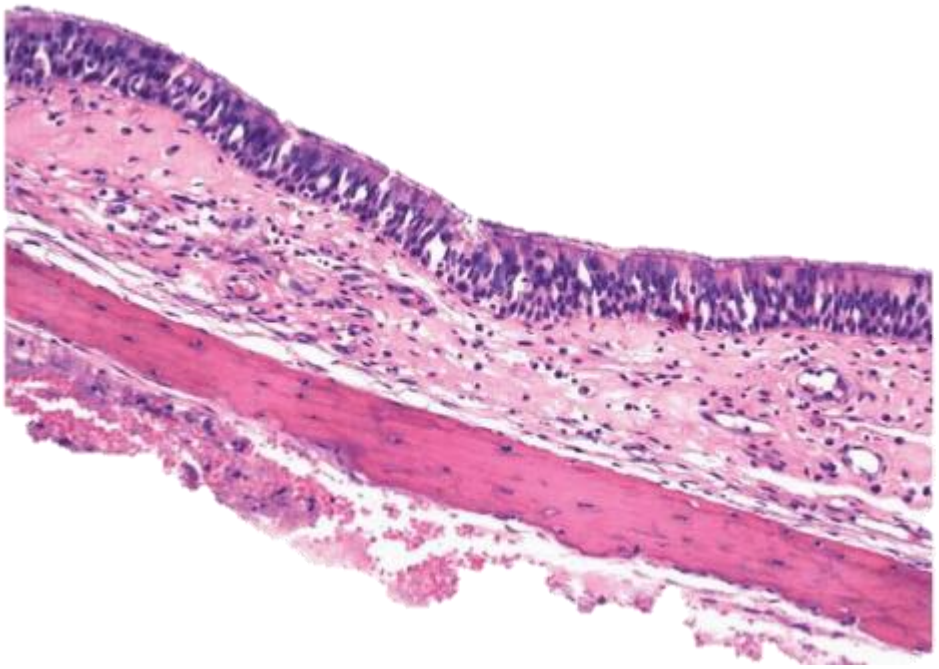
Other changes include the formation of larger fibrous bundles, reduced levels of hyaluronic acid, dehydration, altered crosslinking and the production of elastin fibres and proteoglycans within the matrix. [93] In normal wound healing process, the wound fades due to reduction of vascularity and shrinks in size due to contraction of myofibroblasts [112-114].

1.2.2 Pathophysiology of adhesion and scar formation- general principles

In an abnormal, fibrous wound healing process, the control of tissue repair and regeneration-regulating mechanisms is lost. Clinically, this response is observed as hypertrophy- an enlargement of the cellular components of an organ. [115, 116] The distinguishing feature of a hypertrophic adhesion or scar is the continued proliferation of fibroblasts, with excessive deposition of fibroblast-derived matrix proteins and collagen [117]. Whilst low-level inflammation forms a normal phase of the wound healing process, the formation of adhesions or hypertrophic scars following injury can be exacerbated by an excessive inflammatory processes. [118] Increasing levels of growth factors, inflammatory cytokines and reactive oxygen species (ROS), followed by fibroblast chemotaxis and proliferation, are critical factors in fibrous wound healing [97, 119, 120]. Hypertrophic scar formation can negatively impact the outcome of any surgery, but has an especially well-documented, detrimental impact in abdominal surgery, spinal surgery, vascular surgery, heart surgery and otolaryngology.

1.2.3 Sinonasal mucosa and maladaptive wound healing in the sinus

The sinonasal mucosa acts as a physical barrier to foreign materials and a conditioner for inhaled air. The nasal epithelium lies on the basement membrane, situated on the lamina propria. The pseudostratified columnar epithelium (respiratory epithelium) is composed of four major types of cells: ciliated cells, non-ciliated cells, goblet cells, and basal cells, assuring mucus production and transport, resorption of surface materials, and formation of new epithelial cells [93]. The lamina propria, which equates to the dermis of the integumentary system, consists of two layers of seromucous glands: the superficial layer is situated just underneath the epithelium and the deep layer is under the vascular layer. Just beneath the basement membrane, lymphocytes and plasma cells form a lymphoid layer. The vasculature of the nose is characterized by capacitance vessels. With these vascular specificities, the nasal mucosa can regulate the airflow, adapt the nasal resistance, filter and condition the inspired air and organize the first line of immune defence against airborne pathogens. During the healing process, the ECM of the nasal



mucosa can directly affect the function of growth factors or the expression of their receptors. [93]

Development of post-operative nasal adhesions is the most common complication of nasal and sinus surgery. [121, 122] Rates are typically reported after 3-5 years and range between 15-36%. [122, 123] Adhesions are known to interfere with the normal mucociliary transport, resulting in pooled mucous which is an ideal growth medium for a variety of microbial pathogens. [121] Where they narrow or obstruct ostia, adhesions and synechiae may cause pain from pressure of retained secretions in addition to predisposing to recurrent disease. [93, 121] In those patients requiring revision surgery some authors describe adhesions as being a causative factor of surgical failure in up to 60% of these cases. [123-125]

One pertinent example of maladaptive wound healing leading to revision surgery is recurrent CRS concentrated in the frontal sinus. Due to the narrow confines of the frontal recess, any maladaptive healing in this area may lead to complete stenosis of the frontal sinus. Revision surgery to address frontal sinus stenosis often involves performing a modified endoscopic Lothrop or Draf III procedure [126] to create the maximum possible frontal neo-ostium. Cohort studies have shown that frontal neo-ostiums will stenose by an average of 33% within the first postoperative year. [127] Interestingly, all patients in a prospective study of this treatment course who developed clinically relevant stenosis requiring revision (>60%), developed this stenosis within 12 months of surgery. [127] It is therefore likely that, whilst the severity and extent of this maladaptive wound healing may continue to evolve over months and years, their incidence is determined very early in the postoperative course. [128]

For these reasons, management of adhesions is usually assigned a high priority during revision surgery. Despite this, it is important to recognise that not all adhesions require revision surgery, depending on their density and, perhaps most importantly, their position. In fact, some are intentionally created between the middle turbinate and septum, in order to prevent lateralisation of the middle turbinate and collapse of the middle meatus. It should be noted however that this practice is controversial and has been largely abandoned in otolaryngology due to its detrimental effect of olfaction. [129]

Finally, it is important to acknowledge the role that chronic infection plays in maladaptive wound fibrosis and adhesion formation within the sinus. The sinuses and upper aerodigestive tract confront infectious agents, such as *S. aureus*, on a constant basis. If transcript and protein components of these infectious pathogens persists, as it does in chronic rhinosinusitis with polyposis, the immune response appears to skew to a T-helper 2 (Th2) cytokine profile (i.e., increased IL-4 and IL-13). [130]

Th2 responses activate ECM and collagen deposition, whereas T-helper 1 (Th1) responses inhibit this process; meaning that the two responses have an opposite and mutually-exclusive role in tissue repair. [131] Secondary or repetitive exposure to infectious agents within this Th2 immunoregulated environment exacerbate a collagen deposition and fibrosis, due to a lack of Th1 response. [130]

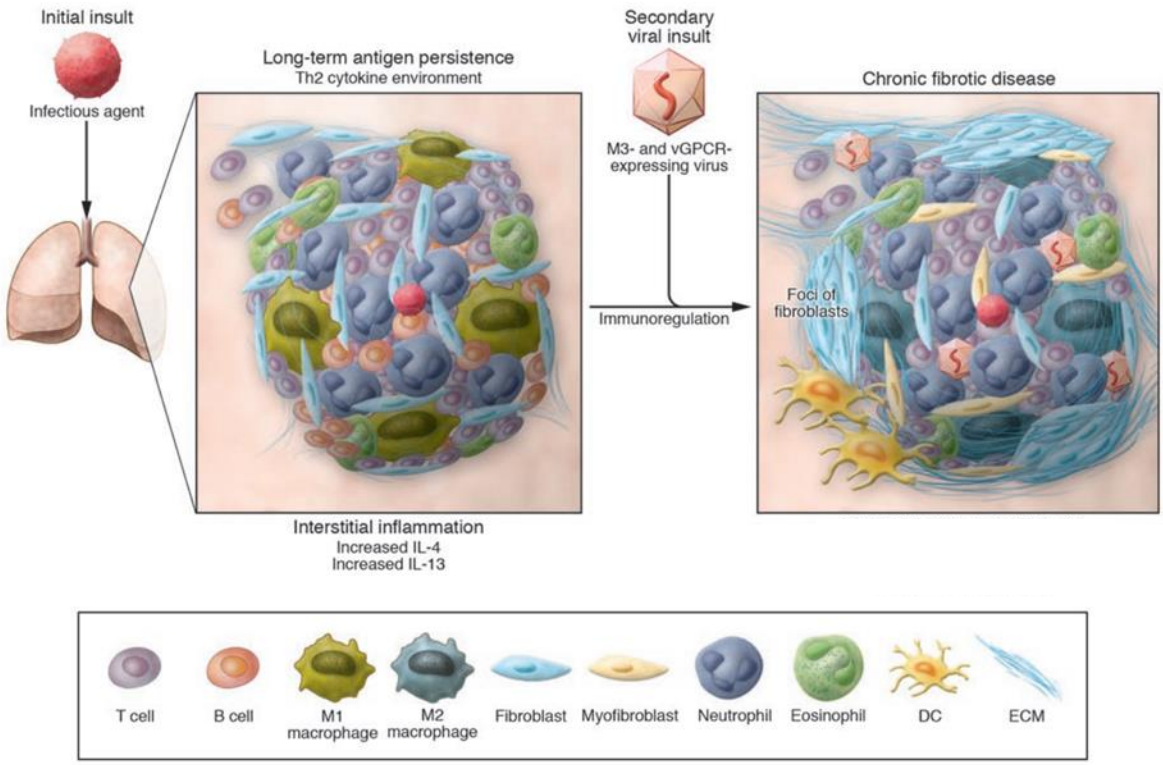


Figure 1.4 A defective host response facilitating chronic fibrosis. From Meneghin and Hogaboam [130] Example of th2-primed IL-4 and IL-13 production leading to chronic fibrosis in the lung and aerodigestive tract.

1.2.4. Existing materials to prevent maladaptive sinonasal healing after FESS

Nasal Packing: Traditionally, nasal packing was used primarily to control ongoing bleeding after sinus surgery, although various proponents also suggested that they played a role in preventing adhesion formation, middle turbinate lateralization and re-stenosis following surgery. Numerous non-absorbable packing agents are still available for use in the postoperative setting, including vaseline soaked ribbon gauze, fingerstall packs, polyvinyl acetate sponge, and various balloon tamponade devices. Unfortunately, removal of non-absorbable nasal packing was also consistently rated by patients to be the most

unpleasant aspect of the surgical experience [132, 133]. Apart from discomfort, other complications associated with removable nasal packing include septal perforation, pack dislodgement, aspiration, toxic shock syndrome, foreign body granuloma, myospherulosis, obstructive sleep apnoea and even death. [134, 135] Pack removal has also been shown to be detrimental to wound healing. Removal from adhered mucosa can cause the surface tissue to be excoriated, leading to further trauma, bleeding and inflammation that results in a fibrinous exudate and potential scar formation. [92] These drawbacks of removable nasal packing have led to the ongoing development and application of absorbable materials that do not require subsequent removal, whilst exerting positive effects on haemostasis, wound healing and middle turbinate support.

Absorbable materials carry out their function either by providing clotting factors or a substrate to stimulate clotting. These packs have been extensively investigated and researched in the ENT literature well before the evolution of endoscopic sinus surgery, with the first use of absorbable biomaterials published in 1969. [136] Over the past two decades, a growing number of RCTs have investigated the effects of emerging packing materials on adhesion formation, crusting, mucosal oedema, inflammation, and cilia regeneration. [4] Some of the more novel materials that have been evaluated against negative control include nonabsorbable Merocel® [137] and absorbable, gelatinous materials such as Floseal®,[138] microporous polysaccharide haemospheres,[139] carboxymethylcellulose,[140] MeroGel®,[141] Sepragel®,[142] and Chitogel®. [143]

Only Chitogel®, Merocel® and Sepragel® have been shown to confer a wound healing advantage over no packing, with lower adhesion rates in their active treatment arms. [137, 142, 143] Chitogel® was also shown, in another RCT, to be associated with

significantly larger sinus ostial sizes at 3 months. [144] Floseal® and carboxymethylcellulose have not been shown to confer any significant benefit on wound healing compared to leaving a cavity unpacked. [140]

Given some of the early perceived benefits of non-absorbable Merocel® in reducing adhesion formation, several RCTs have directly compared emerging absorbable packing materials against Merocel®. These include Floseal®, [145] fibrin sealant, [146] oxidized cellulose, [147]and Nasopore® [148, 149], which were all found to have equivalent results on wound healing and adhesion formation. Contrasting results exist in RCTs comparing Merocel® to the absorbable MeroGel®. Although an RCT by Berlucchi et al. [150] suggested better early and long-term wound healing for MeroGel®, no difference between these agents was observed in two other independent RCTs. [151, 152] An RCT by Shi et al. [153] evaluating a similar hyaluronan-based gel, PureRegen Gel®, observed improved wound healing in terms of adhesion formation, oedema, and crusting when the gel was applied to Merocel®, suggesting it may be the esterified hyaluronic component of MeroGel® that conveys the most benefit.

Although studies by Jameson et al. [138] and Baumann and Caversaccio [154] reported no difference in wound healing or adhesion rates when Floseal® was compared to placebo or Merocel®, concerns have been raised regarding its possible pro-adhesion properties. Studies by Chandra et al. [155, 156] showed histopathological findings of incorporated foreign material within mature synechiae and a subsequent ‘’foreign-body’’ ulcerative reaction. This suggests that Floseal® may incite inflammation and a higher rate of symptomatic adhesion formation. [156]

Stenting: There has recently been an emergence of stents and silicon tubes designed to maintain ostial size and patency following FESS. Some of the proposed advantages include preventing middle turbinate lateralization, acting as a spacer and decrease the clot/mucus filling the middle meatus/ostium, providing a matrix for epithelial migration and acting as an occlusive dressing. [157]

One of the main indications for stent placement is full thickness mucosal disruption, acknowledging that under these circumstances the wound healing process is prolonged by up to 18 months. As such the stent may be required to remain in place for an extended period of time, [158] with several proponents advocating for stents to remain in situ for up to 6 month following surgery. [157, 159] Some authors have found great success in preventing stenosis and meatal collapse, [157] whilst others have found no significant benefit. [160] Potential drawbacks are similar to those of nasal packing- foreign body fibrosis, biofilm formation and the need for painful and potential traumatic removal.

Finally, antibiotic and corticosteroid-eluting stents are a more novel, hybrid system that provide both anatomical support and beneficial drug delivery. They have been put forth as a beneficial, cost-effective treatment to improve postoperative healing in FESS. [161] Experience is early, and most emerging studies in the past few years have been industry-sponsored, making publication bias a potential issue. [161] Specific usage should be at the clinician's discretion taking into consideration various important patient-specific factors.

1.2.5 Experimental models of sinonasal wound healing

In vitro epithelial and fibroblast cell migration post injury has been well described in the literature, using mechanical injuries on a cell monolayer created using a pipette tip, according to a highly reproducible technique. [162-164] This commonly employed wound-

healing assay allows cost-effective study of early mechanisms of injury, i.e. cell migration and proliferation. The use of specialised imaging microscopes that utilise a controlled cell culture chamber is desirable to maximise reproducibility and reliability. The rate of wound closure can then be calculated using a variety of imaging software (e.g. ImageJ, National Institutes of Health, Bethesda, MD, USA) to compare the wound area measured at various timepoints with the initial wound area.

In vivo animal trials have contributed significantly to our understanding of wound healing of the paranasal sinuses and there are a large number of trials that reflect this. The predominant models seen in the literature are the ovine, cunicular and murine models.

Sheep (ovine) models are ideal as they are a large animal model where routine scale sinus surgical techniques can be used, whilst their mucosa is histologically similar to that of humans. [165] Models of bacterial rhinosinusitis have also been developed using blockage of the maxillary sinus ostia, along with *Bacteroides fragilis* inoculation, resulting in a persistent bacterial rhinosinusitis that is easily verifiable and accurately mimics the environment prior to human sinus surgery. [166]

Mouse (murine) and rabbit (cunicular) models have been utilised to a lesser degree, especially in Australia where sheep are more readily available than elsewhere. Nevertheless, rabbits in particular have well-pneumatized sinus cavities, and both their sinonasal anatomy and immunologic reactions are very similar to those of humans, making them a useful animal model for the study of wound healing. [167] A rodent/lagomorph's diminutive size in comparison to sheep and humans is one obvious drawback in assessing mucosal adhesion formation, where surgical techniques must be scaled down and the restricted space may predispose to fibrin bridge formation between

parts of the anatomy. The payoff is that these models are often more far more cost effective and allow for robust effect-analysis, due to increased repeatability and statistical power.

Finally, many human trials have been undertaken in recent years in order to evaluate the potential of various novel agents in reducing adhesion formation after sinus surgery. These typically employ a random allocation model or a self-control model. A patient undergoes bilateral surgery, each side is treated with a different product (usually conventional vs. novel treatment) and the results are compared, safe in the knowledge that many of the host factors will be uniquely and comprehensively controlled for.

1.3 Reactive Oxygen Species

1.3.1 Definition and relationships

The collective term 'reactive oxygen species' (ROS) applies to oxygen-derived molecules which have been reduced with electrons to become highly volatile. The ROS that are capable of independent existence with one or more unpaired electrons (•) are termed oxygen free radicals. An unpaired electron refers to the one that occupies an atomic or molecular orbital 'shell' on its own. The term ''ROS'' encompasses both free radicals and oxygen molecules without any unpaired electrons, which can be termed non-radicals. [168]

Biologically-significant ROS elements include the free radicals- singlet oxygen ($1O_2$), superoxide (O_2•–), hydroxyl (HO•), hydroperoxyl (HO_2•), carbonate (CO_3•–), peroxyl (RO_2•), alkoxyl (RO•) and carbon dioxide radical (CO_2•–). Important non-radicals include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), organic peroxides (ROOH) and hypochlorite (OCl–). Reactive nitrogen species (RNS) are a related group of molecules derived from superoxide via nitric oxide (NO), and include peroxynitrite (ONOO–), peroxynitrate (O_2NOO–), peroxynitrous acid (ONOOH), peroxomonocarbonate ($HOOCO_2$–). [169]

Endogenous cellular ROS can arise from the electron transport chain (ETC) in the inner membrane of the mitochondria during ATP production or from a class of enzymes known as oxidoreductases. ROS acquire electrons from other nearby molecules via a redox reaction, which damages the structure of the latter. There is evidence to suggest that

basal ROS levels are crucial for normal cell functioning and homeostasis and aberrantly low levels of ROS induce cell cycle arrest. [170, 171]

Under physiological conditions, ROS formation is usually in balance with the antioxidant capacity of the host. ROS emission (essentially, production minus scavenging) accounts for 0.25 to 11% of the total oxygen consumed by mitochondria, depending on the animal species and respiration rates. [172] Depending on circumstances, ROS can then initiate a diverse range of cellular responses; signalling pathways involved in cell protection, initiating coordinated activation of mitochondrial fission and autophagy and optimising clearance of abnormal mitochondria and cells to protect against disease spread. [173-175] On the other hand, unregulated ROS activity results in severe cellular damage and unplanned cell death, which can lead to whole organ failure and organism death. [176-178] Mitochondrial oxidative damage increases the tendency of mitochondria to release intermembrane space proteins such as cytochrome c to the cytosol by mitochondrial outer membrane permeabilization, thereby activating the cell's apoptotic machinery. [179] For this reason, the delicate balance between ROS production and scavenging is vitally important.

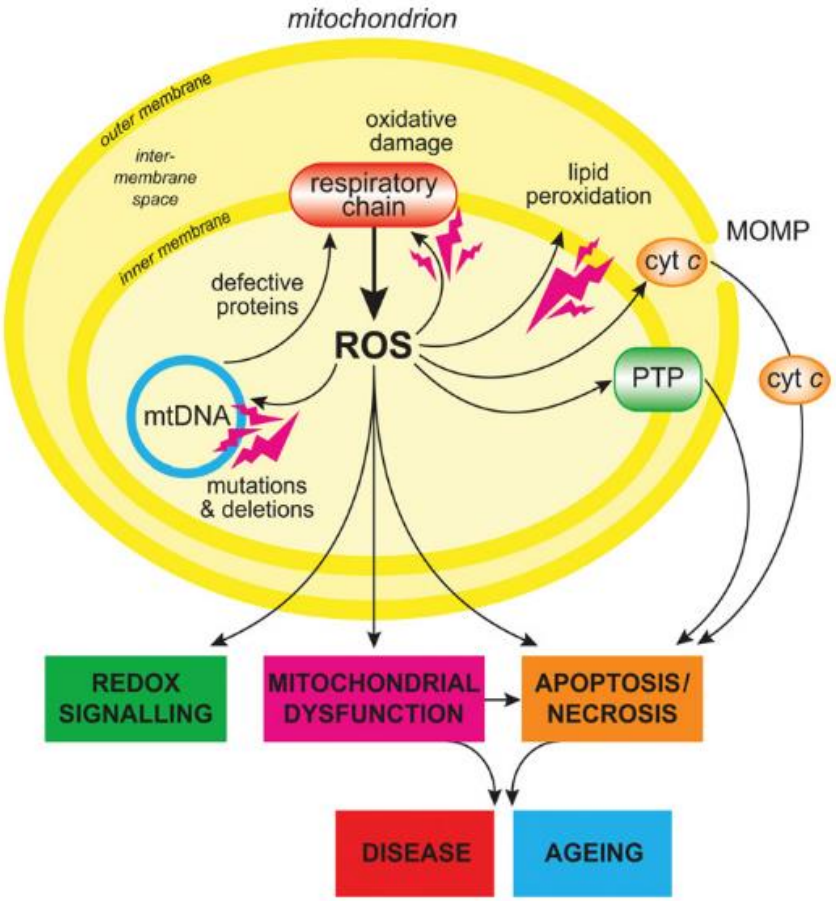


Figure 1.5 Overview of mitochondrial ROS production and downstream consequences

From Murphy 2009 [179]

The homeostatic control of cellular ROS levels (redox state) is the role of a specialist group of proteins known as antioxidants; molecules that have the ability to remove the deleterious effects of ROS by changing their configuration of electrons. In doing so, they nullify their ability to capture electrons from other important molecules, such as DNA, proteins and lipids. [180]

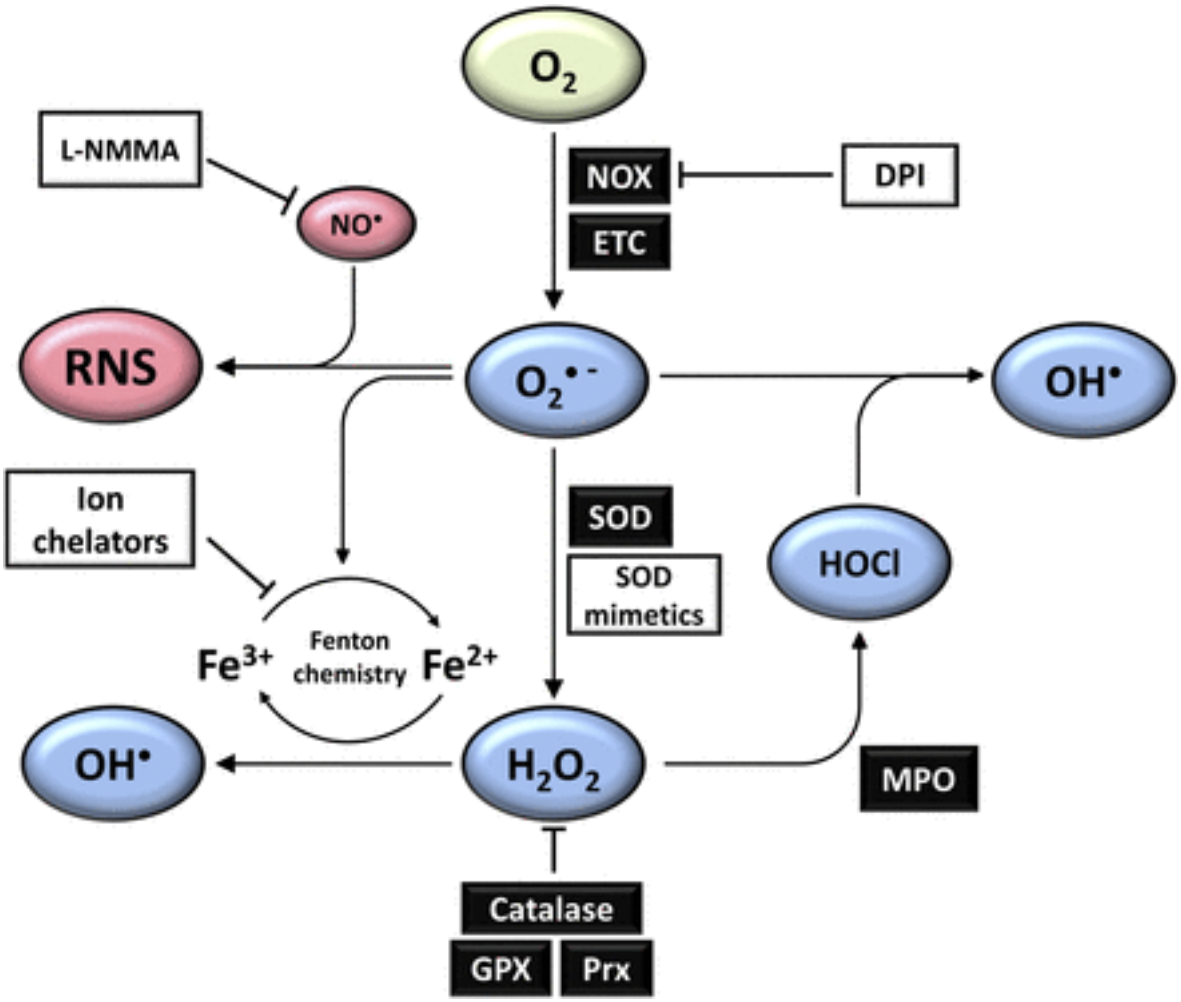


Figure 1.6 Physiologic reactive oxygen species homeostasis network. From Hole [181]. Blue ovals represent ROS, derived from oxygen. Dark boxes represent complexes and enzymes responsible for a given reaction. White boxes represent other interventional mechanisms.

The common pathway of ROS formation begins with the univalent reduction of oxygen, resulting in the formation of superoxide (O_2•−). Superoxide may act as a reductant or an oxidant and is a key molecule in several subsequent physiologic reactions. Most of the superoxide generated *in vivo* is converted into hydrogen peroxide (H_2O_2) primarily by the actions of superoxide dismutases, which exist in cytosolic (SOD1), mitochondrial (SOD2), and extracellular (SOD3) isoforms. Notably, the SODs are the only enzymes that interact

with superoxide specifically. For this reason, they control the levels of ROS and RNS and are regarded as the key regulators of downstream ROS signalling.

Hydrogen peroxide (H_2O_2) however, is tightly regulated by several mechanisms, including the inhibitory influence of catalase, the glutathione peroxidase (GPX) system, and peroxiredoxins (Prx). [181] H_2O_2 may react with myeloperoxidase (MPO) during an immune response to form highly toxic hypochlorous acid (HOCl), which may in turn react with superoxide to form hydroxyl radicals. Hydroxyl radicals are also formed from H_2O_2 by Fenton chemistry, which may occur in the presence of free metal cations such as iron (Fe) or copper.

RNS may be formed wherever superoxide and nitric oxide (NO•) are colocalized, with the proximal species being peroxynitrite (ONOO−). [181] The network of ROS and RNS production can be disrupted or biased in the presence of various compounds such as diphenyleneiodonium (DPI), ion chelators like deferiprone that terminate Fenton chemistry cycles, and l-arginine analogues such as l-monomethyl arginine (L-NMMA), which inhibit nitric oxide synthase.

In addition to proteinaceous ROS control, cells can also utilise important non-enzymatic metabolites as small antioxidant molecules. These include ubiquinone, vitamin C, vitamin E, β-carotene, glutathione, bilirubin, α-tocopherol, nicotinamide adenine dinucleotide phosphate (NADPH) and urate. Moieties with a metal ion capable of oxidation/reduction reactions, such as transferrin and ferritin, also possess a ROS-scavenging capability. [182, 183]

1.3.2 ROS and cell signalling

In light of the fact that many proteins respond to oxidative stress, cells have evolved to utilise ROS for conveying signals to one another. ROS signalling has been implicated in many vital cellular functions associated with cell proliferation, differentiation, migration, immune response, cell senescence and death; as well as a range of inherited and acquired pathologies; ischaemic heart disease, atherosclerosis, neurodegenerative disease, malignant transformation, diabetes, rheumatoid arthritis and aging. [174, 184-190]

When ROS are released and reach a given concentration, they may proceed to trigger mitochondrial permeability transition pores and inner membrane anion channels within individual mitochondria of intact cell systems. The activation of these channels causes intra- and inter-mitochondrial changes related to the new redox balance, which in turn results in further ROS release. The result is an amplified ROS signal which, depending on total ROS levels, may result in a variety of downstream sequelae. This regenerative cycle of mitochondrial ROS formation and release is termed ROS-induced ROS release (RIRR). [191]

The discovery of RIRR has re-framed the mitochondrion as an organelle capable not only of producing energy, but also of facilitating important signalling mechanisms through redox [191], electrical [192], and Ca^{2+} [193] conduction, in a manner analogous to the calcium-activated reaction triggering calcium release from the sarcoplasmic reticulum. [191, 193]

1.3.3 ROS and wound healing

The role of ROS in the pathogenesis of cell death and tissue damage on undamaged cell monolayers also has significant implications for its role in wound healing [194-196]

Various models of wound healing have demonstrated that the most potent ROS in wound-healing and postoperative inflammation is hydrogen peroxide (H_2O_2). [197] This is a result of some fundamental properties; it is easily synthesised by the body, easily degraded, present within all types of cells, more stable than its radical counterparts and, most importantly, it is a small uncharged molecule that can diffuse freely through membranes and tissues. Moreover, it is the least likely ROS to react indiscriminately with neighbouring molecules. [197-199]

In vitro studies have demonstrated that a 10µM concentration of H_2O_2 stimulates the proliferation of human fibroblasts and vascular endothelial cells, whilst also acting as a chemoattractant to inflammatory cells, independent of any blood-bound signalling components. [200-202] This effect has also been seen *in vivo*, as scavenging H_2O_2 after abdominal surgery significantly inhibited postoperative adhesion formation. [203] At 100µM, H_2O_2 promotes angiogenesis by stimulating vascular endothelial growth factor (VEGF) [204] and chemotaxis of keratinocytes in keratinizing epithelium. [205] At 500µM, H_2O_2 stimulates the production of macrophage inflammatory protein-1α, a chemotactic ligand for monocytes, macrophages, neutrophils, eosinophils, basophils and lymphocytes. [200, 206]

In vivo studies utilising wounded dorsal fins of zebra fish demonstrated that H_2O_2 propagates along the wound margin at a decreasing concentration gradient within minutes of epithelial injury. [207] In addition, H_2O_2 was shown to be the key signal for

haemocyte chemotaxis towards the wound. [208] Studies using *Drosophila* showed that calcium, released from the wound edge, travels distally in waves and triggers the release of more H_2O_2 for haemocyte attraction [209] and in *C. elegans* it was shown that H_2O_2 may also link further ROS release to this calcium release. [210] Inhibition of ROS also has a clear cellular impact, preferentially slowing the migration of fibroblasts across wounds and decreasing their collagen production, thus leading to a lower risk of collagen surplus and adhesion formation. [164]

1.3.4 Modulation of ROS by antibiotics

Recently, it has been demonstrated that commonly prescribed classes of bactericidal antibiotics, irrespective of their drug-target interactions, induce oxidative damage via disruption of the ETC. [211, 212] This effect is especially evident at complexes I and III in the mitochondria, [213] where bactericidal antibiotics have been shown to inhibit activity by 16 to 25% (complex I) and 30 to 40% (complex III) compared to untreated samples, leading to an increased 'leakage' of electrons and formation of ROS. Bacteriostatic antibiotics however, such as the tetracyclines and macrolides, exhibited only 5 to 10% inhibition.

Mitochondria-derived ROS are unique in that they can activate the NLRP-3 (NOD-like receptor pyrin domain containing 3) inflammasome. [214] Kohanksi and colleagues found evidence of this in human nasal epithelial cells treated with bactericides [195] suggesting that antibiotic-derived ROS are likely originating from the mitochondria, rather than extra-mitochondrial oxidoreductase activity. To confirm this theory, Kalghati and colleagues [196] treated cells devoid of a functional ETC with bactericidal antibiotics and found no

difference in ROS production when compared to untreated cells. In contrast, there was a significant increase in ROS production stimulated by bactericidal antibiotic treatment in normal cells, further suggesting that the mitochondrial ETC is the major source of bactericidal antibiotic–induced intracellular ROS.

To explain this observation, many have drawn a link to the fascinating theory that mitochondria had a bacterial evolutionary origin. According to this ''endosymbiotic theory'', mitochondria originated from free-living, aerobic bacteria that integrated themselves into amitochondrial eukaryotes at some point in their evolution. [215] They provided this cell with a reliable source of energy, whilst being afforded protection within its membrane. It is likely then, that antibiotics target mitochondria and mitochondrial components in a similar fashion to targeting bacteria. Several *in vivo* studies in mammalian systems have found evidence of these parallel antibiotic-target interactions in both bacteria and mitochondria. [216-219] In terms of specific examples; aminoglycosides target both bacterial [220] and mitochondrial ribosomes, [217] quinolones target both bacterial gyrases [221] and mtDNA topoisomerases, [216] and β-lactams inhibit both bacterial cell wall synthesis [222] and mitochondrial carnitine/acylcarnitine transporters. [218]

Previous work has shown that mammalian cells can be damaged by antibiotic treatment, but these are often applied at concentrations considerably higher than those applied clinically. At these high concentrations, select antibiotics inhibited cell growth and metabolic activity across a variety of human cell lines, in addition to impairing mitochondrial function *in vitro*. [223, 224] Nonetheless, even at lower doses, it is feasible that the oxidative stress induced by bactericidal antibiotics is at least partly responsible

for the adverse effects associated with these drugs. Some prominent examples include aminoglycoside-induced hearing loss [225] and nephrotoxicity [226], as well as fluoroquinone-associated tendinopathy. [227]

The chronic inflammatory state seen in CRS may also increase cellular susceptibility to antibiotic-mediated ROS, particularly if they are unwittingly prescribed in an inappropriately long or repeated fashion during recalcitrant CRS. Less commonly, some patients with compromised antioxidant defence systems or those genetically predisposed to developing a mitochondrial dysfunction disease might be particularly vulnerable to any bactericidal antibiotic collateral damage. [228]

This emerging body of evidence into the mechanisms underlying bactericidal antibiotic side-effects offer a deeper insight into the breadth of their influence and offer new strategies and opportunities to curb their prevalence. For example, antioxidants targeted at mitochondrial ROS have already shown potential in preventing aminoglycoside-induced hearing loss in cultured organ of Corti cells. [229]

The possibility of using an antioxidant to alleviate the deleterious effects of ROS have previously been explored using nasal mucosa. [196, 230] Much of the experimentation that has been undertaken in this area has utilised the antioxidant N-Acetyl Cysteine (NAC), largely because it is an FDA-approved antioxidant that is well tolerated by patients and commonly used to buffer extraneous intracellular ROS in mammalian systems. In a study by Kalghati and colleagues [196], MCF10A cells were pre-treated with NAC for 2 hours, followed by bactericidal antibiotic treatment for 6 and 96 hours. NAC pre-treatment reduced bactericidal antibiotic–induced ROS levels and restored mitochondrial membrane potential to levels seen in untreated cells after four days of antibiotic

treatment. Furthermore, NAC restored basal respiration and maximal respiratory capacity of bactericidal antibiotic–treated cells to near normal levels and alleviated bactericidal antibiotic–induced DNA damage.

One concern is that combining antioxidants and antibiotics theoretically has the potential to reduce the antimicrobial efficacy of the antibiotic; particularly bactericidal antibiotics, given that antibiotic-induced ROS formation is thought to contribute to bacterial killing. [211, 231] Indeed, non-targeted antioxidants, such as the ROS-quenching antioxidant thiourea, have been shown to reduce the bacterial killing efficacy of bactericidal antibiotics. [231, 232] In direct contrast, there is emerging evidence that antioxidants specifically engineered to target the mitochondrial ETC may augment the activity of existing antibiotics [229] by causing bacterial membrane disruption– these are discussed in more detail below.

3.4 Ubiquinone, the electron transport chain and reactive oxygen species

Ubiquinone (also known as coenzyme Q or CoQ) is a mobile component of the mitochondrial ETC, where it acts as both an anti- and pro-oxidant in its various states of reduction. Ubiquinone can exist in three different redox states: fully oxidized (ubiquinone, UQ), partially reduced (ubisemiquinone, $UQ^{\bullet -}$, a free radical), and fully reduced (ubiquinol, UQH_2). [233] The ability of ubiquinol to undergo reversible redox cycling between these three states makes it a highly versatile carrier molecule, and a vital component of the ETC.

In the inner mitochondrial membrane, ubiquinol transfers electrons from complexes I and II to complex III. This can be demonstrated *in vitro* through chemical extraction of ubiquinol from mitochondrial membranes, which results in the loss of NADH oxidase and

succinate oxidase activities (a measure of complex I and II activity respectively) until ubiquinol is reincorporated into the membrane. [234] The long sidechain of ubiquinol is anchored in the central hydrophobic portion of the membrane, with the benzoquinone head moving through the hydrophilic regions. [235] All redox components diffuse laterally and randomly in the inner mitochondrial membrane, and the transfer of electrons occurs randomly whenever the complexes and the two mobile electron carriers (ubiquinol and cytochrome c) collide with one another. Adding phospholipids to the inner mitochondrial membrane to dilute its components results in decreased rates of electron transfer from complex I or II to complex III, while subsequent addition of ubiquinol restores electron transfer substantially. [236] This suggests a direct influence of ubiquinol concentration on electron transfer. Being much smaller, ubiquinol should diffuse faster than the bulkier ETC complexes; however, it remains unclear whether ubiquinol diffusion is rate-limiting for electron transport. Even so, ubiquinone is still a large molecule that does not easily penetrate cells. Its long sidechains are necessary for its function in the membrane and make it highly lipophilic and difficult to absorb. This poor bioavailability of exogenous ubiquinol likely accounts for its largely unsatisfactory efficacy in humans when given as an unaltered supplement [237], presenting a need in clinical practice for more targeted therapies.

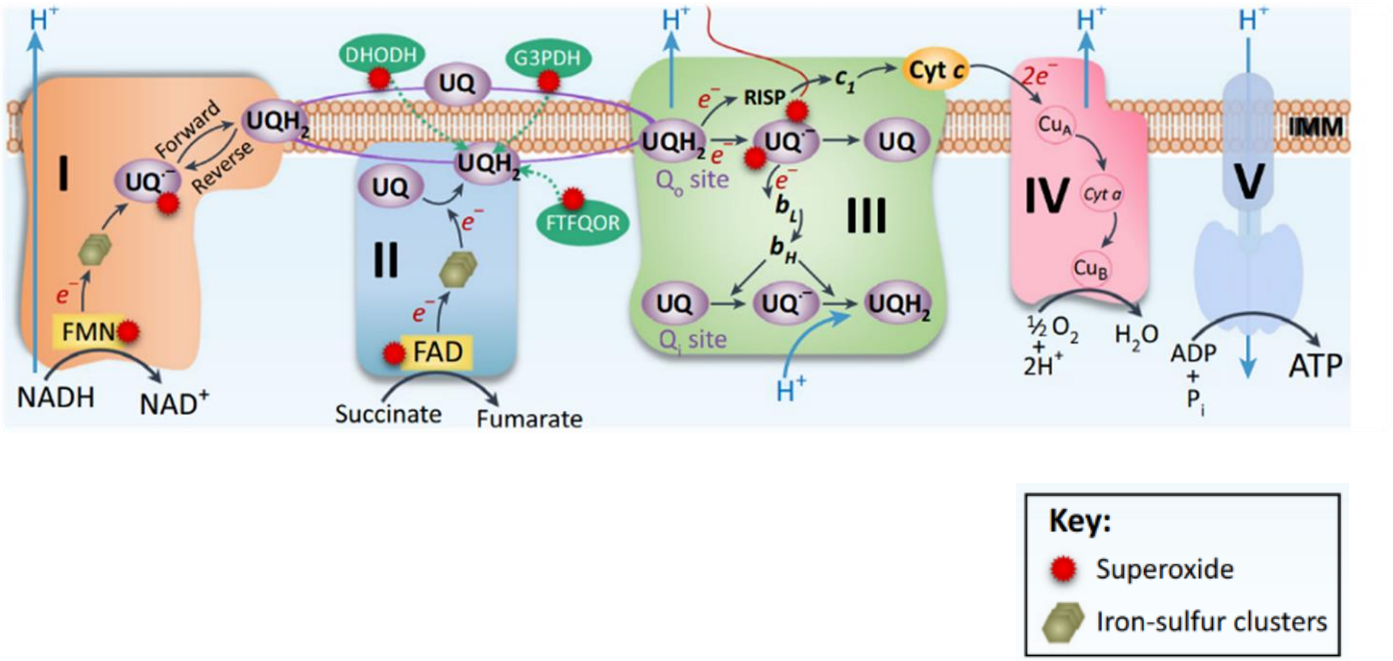


Figure 1.7 Functions of ubiquinone (UQ), ubisemiquinone (UQ•–) and ubiquinol (UQH_2) in the mitochondrial electron transport chain.

From Wang and Hekimi [233] In normal forward electron transfer, ubiquinone (UQ) accepts electrons from complexes I and II and transports them individually to complex III. At complex III, the 'Q cycle' allows pumping of protons from the matrix into the intermembrane space. This involves two distinct ubiquinone binding sites. Ubiquinol (UQH_2) is reduced at the Q_o site, passing one electron to cytochrome c (cyt c) and the other down to the Q_i site, where the electron is either given to a bound UQ during the first cycle, forming ubisemiquinone (UQ•–), or to a bound UQ•– already generated during this first cycle. Oxidized UQ formed at the Q_o site and UQH_2 formed at the Qi site after completion of the 'Q cycle' are free to diffuse out into the ubiquinone pool. (UQ) also accepts electrons from several non-respiratory chain dehydrogenases, including the mitochondrial glycerol-3-phosphate dehydrogenase (G3PDH), dihydroorotate dehydrogenase (DHODH), and electron transfer flavoprotein oxidoreductase (ETFQOR). As electrons are transported, they may leak to oxygen, forming the ROS superoxide ($O^{2}\bullet^{-}$).

1.3.5 Mitochondrially-targeted antioxidants

Mitochondria are a major source of ROS, with superoxide generation resulting from single-electron premature reduction of oxygen by electrons moving through the ETC in its inner membrane. [179] The mitochondrial membrane potential drives natural cationic substances into mitochondria; a property that can be utilized to help man-made drugs penetrate the organelle. [191] This approach uses cationic, lipophilic compounds constructed in a way to delocalize charge over a set of coupled double bonds. [238] Due to the significant negative charge within the mitochondrial interior, these cations can accumulate to a level that exceeds extramitochondrial concentration by about three orders of magnitude.

This approach serves a dual purpose: to deliver desired compounds to the mitochondrial interior [239-241] and, on the other hand, to help discriminate whether a given process is mitochondrial or non-mitochondrial in nature. In the first case, *in vitro* data and animal experiments have suggested the possible beneficial effect of mitochondria-targeted antioxidants (such as mitoquinone, SkQ1, SkQR1, and others) in the treatment of a great number of model systems associated with oxidative stress. These include stroke, arrhythmias, ischemia/reoxygenation, chemical toxicity, infection, inflammation, and some inherited and acquired age-related diseases: Alzheimer's, diabetes, hepatitis and metabolic syndrome. [191, 242-255] These investigations have also spread to the field of otolaryngology, where mitochondria-targeted antioxidants have shown potential in preventing aminoglycoside related hearing loss. [229, 256]

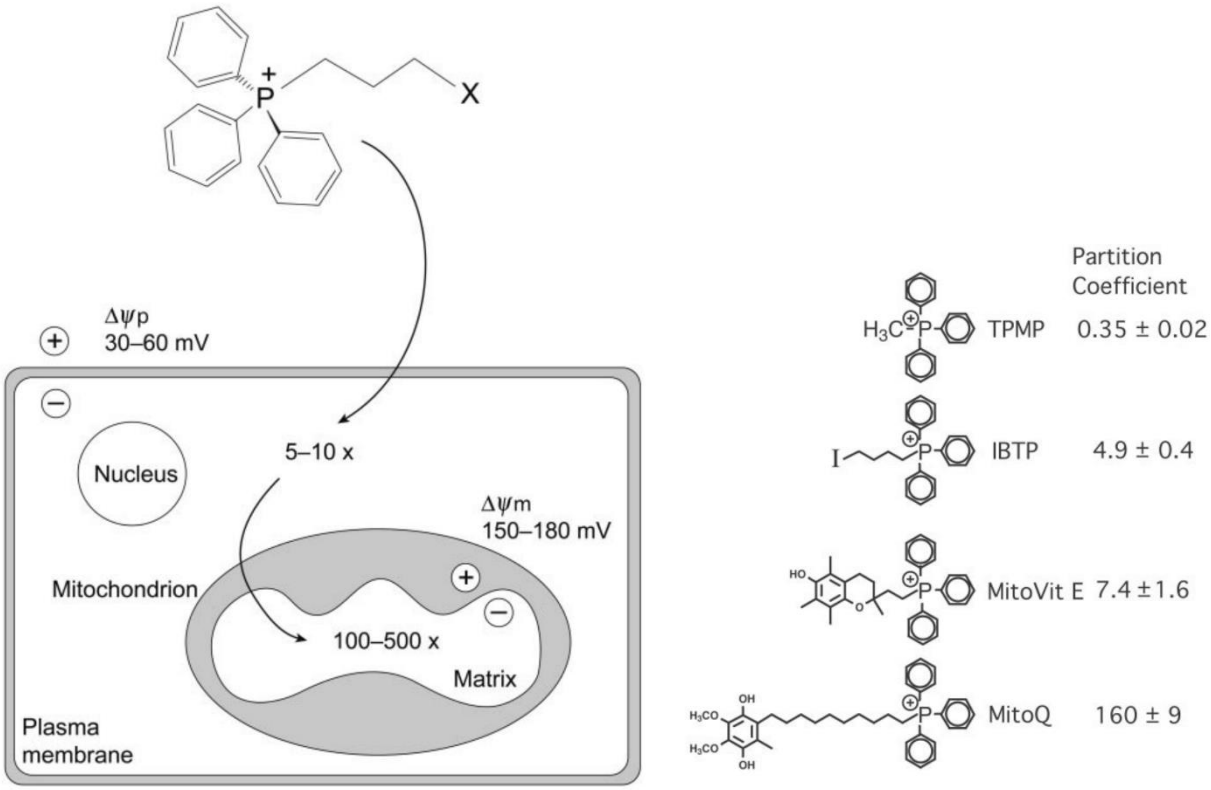


Figure 1.8 Uptake of alkyl-triphenylphosphonium cations by mitochondria within cells.

From Smith [243]. The lipophilic triphenylphosphonium cation is covalently attached to a biologically active molecule (X) such as an antioxidant or pharmacophore. The lipophilic cation is accumulated 5- to 10-fold into the cytoplasm from the extracellular space by the plasma membrane potential (30-60mV) and then further accumulated 100- to 500-fold into the mitochondrial matrix by the mitochondrial membrane potential (150-180mV). As these lipophilic cations pass directly through the lipid bilayer, they do not utilize specific uptake systems and have the potential to distribute to mitochondria in all organs, including the brain.

Apart from the practical issue of using mitochondria-targeted chimeric compounds as potential therapeutic agents, their specific effects uncovered the fundamental role of mitochondria and mitochondrial ROS in the onset and propagation of different pathologies. ROS originating from mitochondrial rather than from other intracellular sources, when they exceed the homeostatic level, are emerging as some of the most pathogenic factors in the inflammatory milieu. [257-261] The requirement of mitochondria-derived ROS for the propagation of a ROS-driven pathological cell death has received support from experiments wherein 1) extramitochondrial ROS induced a secondary intramitochondrial ROS release, and 2) both of these processes were prevented by specific mitochondria-targeted antioxidants. [262] In this context, targeted normalization of ROS levels may be exponentially beneficial in preventing ROS-related pathologies.

1.3.6 Mitochondrially-targeted antioxidants as antimicrobials

The antibacterial action of MTAs has been attributed to the activity of their alkyl salts [263, 264] which have the ability to disturb the bacterial membrane. [265] In particular, these salts exert their membrane-perturbing effects when bound to quaternary phosphonium compounds, [266] in a similar fashion to that of quaternary ammonium sterilizing agents, such as cetrimide. [267] Such action leads to a generalized and progressive leakage of cytoplasmic materials to the environment, causing bacteria to lose osmoregulatory capability.

MTAs bactericidal action may also involve suppression of bacterial bioenergetics, by collapsing bacterial membrane potential via protonophorous uncoupling. [255, 268-270] Protonophores uncouple oxidative phosphorylation by facilitating proton transfer across

lipid bilayers. Whilst MTAs are not protonophores, they can perform a protonophore-like action when combined with fatty acids, [270] which are abundant in cellular and mitochondrial membranes. MTA import by bacteria is driven by the electrical potential gradient, negative inside bacterium. As MTAs are exported from the cell in the form of a cation/anion pair, they provide a mechanism for outward transport of the anionic fatty acids. These ions are then protonated and imported back into the cell, with the subsequent deprotonation to anion and H+ on the inner surface of the inner bacterial membrane causing a net reduction in the negative charge of the inner matrix that interferes with ATP synthase activity.

In a recent study by Nazarov and colleagues, an MTA named SkQ1 demonstrated very efficient antibiotic activity, inhibiting growth of *B. subtilis*, *S. aureus, P. phosphoreum, R. sphaeroides* and *Mycobacterium sp*. at micromolar concentrations. Importantly, SkQ1 acted as a bactericidal agent at concentrations higher than MIC, whereas it acted as a bacteriostatic agent under the conditions when its level was not sufficient to cause the collapse of membrane potential. Values of the MIC of SkQ1 for *S. aureus* were lower than (or comparable to) those of several conventional antibiotics (including vancomycin, azithromycin, chloramphenicol, streptomycin, and kanamycin). [271]

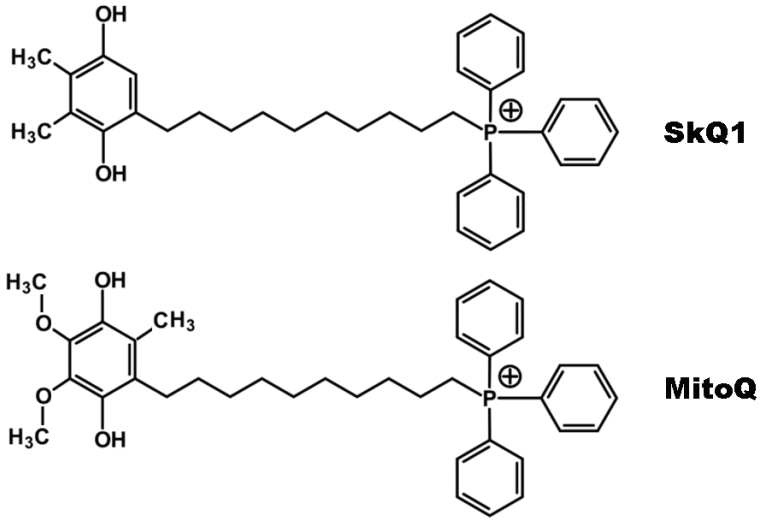


Figure 1.9 Chemical structures of the MTAs SkQ1 and mitoquinone (MitoQ)

From Nazarov et. Al. (supplementary materials) [255]

Whilst evidence for their efficacy against gram positive bacteria is growing, some studies have shown that MTAs are less effective against gram negative and anaerobic bacteria. [229, 255] One explanation may be the presence of specific transporters, such as AcrAB-TolC in some strains of *E. coli* that actively facilitates efflux of MTAs. [255] However, unlike other antioxidants, the activity of antimicrobials against gram negative pathogens like *Pseudomonas aeruginosa* and *Haemophilus influenzae* have have been shown not to be compromised by MTAs at pharmacological levels [229]. This means this issue of interference could potentially be circumvented by combination therapy of MTAs with appropriate antimicrobials targeting gram negatives and anaerobes.

1.3.7 Human studies using mitoquinone

Antipodean Pharmaceuticals Inc. first developed the mitochondrially-targeted antioxidant mitoquinone in New Zealand in 2001, [239] and it has since become the most intensely studied MTA in the scientific literature. On that basis, it has started moving into the human trial sphere where, as of December 2019, there have been eleven mitoquinone

human trials registered with clinicaltrials.gov (3 completed, 7 ongoing, 1 terminated for poor recruitment).

For a stable oral formulation it was found beneficial to make mitoquinone with the methanesulfonate counter anion and to complex this with cyclodextrin. This preparation was readily made into tablets that passed conventional animal toxicity testing. The oral bioavailability was determined at about 10% and major metabolites in urine were glucuronides and sulfates of the reduced quinol form along with demethylated compounds. In human Phase 1 trials mitoquinone showed good pharmacokinetic behaviour with oral dosing at 80 mg (1 mg/kg) resulting in a plasma maximal concentration of 33.15 ng/mL and after ~1h. [247]

Mitoquinone was first trialled to see if it could slow disease progression of Parkinson's Disease (PD), due to multiple lines of evidence pointing to mitochondrial oxidative stress as a potential pathogenic cause. [272] In this 13-centre study in New Zealand and Australia 128 newly diagnosed untreated patients with PD were enrolled in a double-blinded study of three treatment arms (mitoquinone 40 mg/day, mitoquinone 80 mg/day and placebo) to see whether, over 12 months, mitoquinone would slow an improvement in the Unified Parkinson Disease Rating Scale. This study showed no difference between mitoquinone and placebo on any measure of PD progression. [272] The explanation put forth was that, by the time Parkinsonism is clinically evident, approximately 50% of dopaminergic neurons are lost. It is possible that at diagnosis the fate of the remaining neurons is already determined and neuroprotection at this stage cannot prevent their death. Insufficient brain penetration was also considered as a possible mechanism, though in a

rodent model mitoquinone protected against substantia nigra damage, preserved locomotor activity and dopamine content as well as decreasing mitochondrial markers of oxidative damage. [273] While there was no therapeutic efficacy in the PD study, this body of work provided some important safety data for the long-term administration of mitoquinone in humans and demonstrated that it can be safely administered as a daily oral tablet to patients for at least a year.

The second mitoquinone human trial is the CLEAR trial on chronic hepatitis C virus (HCV) patients by Gane and colleagues. [246] HCV patients who were unresponsive to conventional HCV treatments were selected as subjects, because in this group of patients there is evidence for increased oxidative stress and subsequent mitochondrial damage playing an important role in ongoing liver damage. Therefore, the effect of oral mitoquinone on serum aminotransferases and HCV RNA levels in HCV infected patients was assessed in a double-blinded, randomized, parallel design trial of three treatment arms (mitoquinone 40 mg/day, mitoquinone 80 mg/day and placebo) in patients with a documented history of chronic HCV infection for 28 days. Both treatment groups showed significant decreases in serum alanine transaminase (ALT) from baseline to treatment day 28. There was no effect of mitoquinone on viral load, indicating that mitoquinone only affected oxidative liver damage associated with HCV infection, and did not inhibit the viability or reproduction of the virus in the same way it affects bacteria. These data suggest that mitoquinone can reduce liver damage in HCV infection. More generally, this study is the first report of a potential clinical benefit from the use of mitochondria-targeted antioxidants in inflammatory disease of humans. Coupled with the 1 year's safety data for mitoquinone from the Parkinson's study, this suggests that the efficacy of

mitoquinone for other chronic conditions that are thought to involve mitochondrial oxidative damage, such as CRS, are a worthy undertaking.

The latest study to date was published in the USA in 2018 and showed that supplementation with mitoquinone improved vascular endothelial function in healthy late middle-aged and older adults, by reducing the tonic suppressive effects of excessive mitochondrial-specific reactive oxygen species. [274] Mitoquinone also reduced aortic stiffness in individuals with age-related arterial stiffening. Twenty healthy older adults (60–79 years) with impaired endothelial function (brachial artery flow-mediated dilation <6%) underwent six weeks of oral supplementation with mitoquinone (20 mg/day) or placebo in a randomized, placebo-controlled, double-blind, crossover design study. Brachial artery flow-mediated dilation was 42% higher after mitoquinone vs. placebo ($P<0.05$), and this improvement was associated with amelioration of mitochondrial reactive oxygen species-related suppression of endothelial function. Aortic stiffness (carotid-femoral pulse wave velocity) was lower after mitoquinone vs. placebo ($p <0.05$) in participants with elevated baseline levels (carotid-femoral pulse wave velocity >7.60 m/s, n=11). Plasma oxidized low-density lipoprotein, a marker of oxidative stress, also was lower after mitoquinone vs. placebo ($p <0.05$). Participant characteristics, endothelium-independent dilation (sublingual nitroglycerin) and circulating markers of inflammation were demonstrably comparable at baseline. These findings in humans extend earlier preclinical observations and suggest that mitoquinone and other therapeutic strategies targeting mitochondrial reactive oxygen species may hold promise for treating age-related vascular dysfunction.

The remaining prospective trials listed follow on in a similar vein, targeting neurological and vascular conditions such as Alzheimer's, Multiple Sclerosis and peripheral artery insufficiency: (https://clinicaltrials.gov).

Importantly, from the pharmaceutical development viewpoint, no severe adverse events were reported in any of the studies. The most common treatment-related adverse event was mild nausea that was dose-dependent. As there was no dose dependence for efficacy demonstrated in Gane's HCV study [246], future studies may be able to limit nausea while retaining efficacy by lowering the mitoquinone dose.

Summary of Literature Review

Despite CRS being a very common disease, there is still much left to unravel with regards to its aetiology and the avenues available to reduce its disease burden. Recent developments in medical therapy and surgical techniques have achieved much, but these are not without their own pitfalls.

Some of the most common adverse outcomes after sinus surgery relate to poor wound healing, driven at least in part by the overactive inflammatory process. Reactive oxygen species (ROS) are known to play a significant role in this inflammatory milieu and exert their influence on wound healing in an ever-evolving fashion. All of this is compounded by the presence of fastidious bacteria, provoking further responses from the host immune system. Antibiotics are often administered to patients to try and combat these bacteria, and these have been shown to affect ROS production of intact nasal cell monolayers *in vitro.* Their effect in the setting of active wound healing, however, remains unclear.

On the topic of antibiotics: biofilms comprising a fastidious reservoir of bacteria are frequently identified in patients with recalcitrant infection. Systemic antibiotics are often insufficient to remedy these biofilms, provoking the need for alternate treatments. Antioxidants are one class of therapies that have shown promise in treating and preventing biofilms. The recent development of antioxidants targeted directly at the ROS-producing inner mitochondrial matrix of the cell presents an exciting and novel opportunity to harness this therapy in the sinuses.

The majority of existing human trials using MTAs have utilised oral formulations and have shown a robust safety profile. Development of topical therapies that utilise MTAs presents

a unique opportunity to address the environmental and host factors that drive CRS persistence in sinonasal mucosa after sinus surgery, in a more directed and measured fashion than existing therapies.

A number of research questions have arisen from this literature review:

1. Do the antibiotics commonly prescribed in otolaryngology contribute significantly to ROS production and how does this affect cellular migration in the setting of active wound healing? This information may prove useful in guiding postoperative antibiotic therapy in sinus surgery, with the ultimate aim being a reduction in the incidence of postoperative adhesions.
2. Are mitochondrially-targeted antioxidants an effective alternative treatment against the biofilms formed by pathogenic bacteria within the sinuses? How does their efficacy against planktonic bacteria translate into anti-biofilm activity? Could this be a potential new treatment for sinus biofilms that concurrently addresses the high oxidative stress and inflammation synonymous with CRS?
3. Do mitochondrially-targeted antioxidants have a role in the inhibition of adhesion formation in sinonasal mucosa? Can a link with ROS be elucidated by closely observing sinonasal fibroblasts and epithelial cells treated with these agents?
4. Are these treatments safe? What is the dose-response relationship? Given that a certain level of ROS are vital for the normal house-keeping functions of the cell, can we maintain the 'delicate balance' between safety and efficacy when employing these agents in the sinuses?

What follows is an attempt to address these research questions and, as always, develop many more.

Studies to be Performed

1. Determine the effect of commonly prescribed antibiotics in sinus surgery on ROS production and human sinonasal cell migration.
2. Determine the effectiveness of mitochondrially-targeted antioxidants against *S. aureus* biofilms.
3. Determine the safety and efficacy of mitochondrially-targeted antioxidants for reducing sinonasal adhesion formation *in vitro*
4. Use an *in vivo* animal model to assess the safety and efficacy of using mitochondrially-targeted antioxidants to treat surgical wounds infected with biofilms.

Statement of Authorship

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Contribution to the Paper	Project design, experimental work, data collection and analysis, manuscript preparation		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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Antibiotics Affect ROS Production and Fibroblast Migration in an *In-Vitro* Model of Sinonasal Wound Healing

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Keywords: antibiotics, ROS, wound healing, fibroblasts

Abstract

Introduction: Antibiotics are often administered to patients perioperatively and have been shown to affect ROS production of nasal cells *in vitro*, but their effect in the setting of active wound healing remains unclear. Reactive oxygen species (ROS) are known to play a significant role in wound healing. This study analyzed a broad array of antibiotics used after sinus surgery to assess their effect on wound healing and ROS production *in vitro*. It was hypothesized that ROS production would be affected by these antibiotics and there would be a negative relationship between ROS activity and cell migration speed.

Methods: Monolayers of primary human nasal epithelial cells (HNEC) and primary fibroblasts were disrupted with a linear wound, treated with 10 different antibiotics or a ROS inhibitor and observed over 36 hours in a controlled environment using confocal microscopy. ROS activity and migration speed of the wound edge were measured at regular intervals. The relationship between the two parameters was analyzed using mixed linear modelling.

Results: Performing a linear scratch over the cell monolayers produced an immediate increase in ROS production of approximately 35% compared to unscratched controls in

both cell types. Incubation with mitoquinone and the oxazolidinone antibiotic linezolid inhibited ROS activity in both fibroblasts and HNEC in association with slowed fibroblast cell migration ($p<0.05$). Fibroblast cell migration was also reduced in the presence of clarithromycin and mupirocin ($p<0.05$). A significant correlation was seen between ROS suppression and cell migration rate in fibroblasts for mitoquinone and all antibiotics except for azithromycin and doxycycline, where no clear relationship was seen. Treatments that slowed fibroblast cell migration compared to untreated controls showed a significant correlation with ROS suppression ($p<0.05$).

Conclusion: Increased ROS production in freshly wounded HNEC and fibroblast cell monolayers was suppressed in the presence of antibiotics, in correlation with reduced fibroblast cell migration. In contrast, HNEC cell migration was not significantly affected by any of the antibiotics tested. This differential effect of antibiotics on fibroblast and HNEC migration might have clinical relevance by reducing adhesion formation without affecting epithelial healing in the postoperative setting.

INTRODUCTION

Healing of the sinonasal mucosa and its underlying matrix is a highly regulated process that occurs after mucosal trauma. Successful healing involves a complex interplay between many different cell types and the molecular counterparts involved in their cell signaling pathways.

Where this process becomes dysregulated, bands of scar tissue termed adhesions may form in the nasal cavity between the remnant mucosal structures. This is a dynamic process, whereby surgically traumatized tissues that are in apposition begin to bind together across loose fibrin bridges, formed during the hemostatic stage of wound healing [93]. Whilst these structures remain highly mobile initially, they will begin permanently adhering to one another as fibroblasts infiltrate the wound and begin depositing collagen. These collagen bands begin to develop on postoperative days 3-5, and continue to evolve until day 14, after which they stabilize [275]. The distinguishing feature of a dense adhesion is the continued proliferation of fibroblasts, with excessive deposition of fibroblast-derived extracellular matrix (ECM) proteins and collagen [276]. The process of postoperative adhesion begins during surgery, and whilst the severity and extent of these adhesions may evolve over weeks and months, their incidence is determined within the first postoperative week [128].

Reactive oxygen species (ROS) are oxygen derived molecules produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria [277]. ROS act within cells to promote migration, whilst also working in nonmigrating cells to influence the behavior of migrating cells through ROS signaling [278]. They normally exist within a delicate homeostasis, regulated by the antioxidant capacity of their host, and play an important role in wound healing and adhesion formation [180, 278, 279]. Whilst ROS production has been noted predominantly in the two hours after injury, their effects on wound healing remain detectable for over 24 hours [280].

It has been proposed that ROS signaling is concentration dependent and that, whilst high ROS levels may induce apoptosis, moderate levels activate the inflammatory response and low levels activate metabolic signaling [281]. Manipulation of cellular ROS has been shown to slow the wound migration of fibroblasts *in vitro* [164, 282] and to inhibit postoperative adhesion formation in animal models of surgery [279].

A significant factor modulating ROS production in the postoperative setting is the presence of antibiotics. Antibiotics are routinely given after sinus surgery, although their role in this setting remains equivocal [4, 69, 283, 284]. Apart from their direct antimicrobial effects, many antibiotics also have immunomodulatory functions and have been shown to affect ROS production. Specifically, bactericidal antibiotics have been shown to increase ROS production, whilst bacteriostatic antibiotics do not [194, 195]. Among the classes of antimicrobials that have been best characterized in the literature are beta-lactams, macrolides and quinolones [195], whilst the effects of other antimicrobials used in otorhinolaryngology, such as tetracyclines and mupirocin, remain poorly understood. To date, such studies have focused on simulating the non-operative treatment of sinus disease rather than a post-operative setting, where ROS may play a more significant or complex role due to fresh mechanical disruption of the tissue.

In the present study, we investigate the cytokinetic effect of antibiotics on sinonasal fibroblasts and epithelial cells that have been exposed to mechanical trauma. Their effect on ROS production was quantified using real-time confocal LASER microscopy. We hypothesized there would be a variable effect on cell migration and ROS production across individual antibiotics, and that there would be a negative relationship between these two effects. This information may prove useful in guiding postoperative antibiotic therapy in sinus surgery, with the ultimate aim being a reduction in the incidence of postoperative adhesions.

MATERIALS & METHODS

Study population

This study was performed in accordance with guidelines approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital and the University of Adelaide (reference HREC/15/TQEH/132). All patients that donated cells gave written informed consent and all samples obtained were anonymized and coded before use. All methods were carried out in accordance with the relevant guidelines and regulations. Patients recruited to the study included those who were undergoing endoscopic sinus surgery for chronic rhinosinusitis (CRS). Exclusion criteria included active smoking, age less than 18 years, pregnancy, systemic immunosuppressive disease and underlying malignancy.

Harvesting and culturing primary human nasal fibroblasts *in vitro*

Sinonasal tissue was biopsied from paranasal sinus mucosa and transferred to a 6-well culture plate with 2 ml Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, UK) supplemented with L-glutamine, 10% Fetal bovine serum (FBS, Sigma, St Louis, USA) and penicillin streptomycin (Gibco, Life Technologies, NY, USA). Every 2-3 days, the tissue was washed gently with 1 ml phosphate-buffered saline (PBS) and medium was replaced with 1.5 ml fresh medium until fibroblasts became confluent after approximately 2 weeks.

Purification of fibroblasts

Once confluent, fibroblasts were washed with 2 ml PBS, trypsinized and collected followed by centrifugation at 400×G for 8 minutes. The supernatant was removed and the pellet resuspended in 1 ml PBS along with 50 µl Dynabeads Epithelial Enrich (Invitrogen, NY, USA). The tube was wrapped in parafilm and placed on a rotor mixer for 20 minutes at room temperature (RT). Supernatant containing fibroblasts were transferred to a T25

tissue culture flask (Nunc, Roskilde, Denmark) and the tube containing the remaining beads discarded. Culture purity was confirmed morphologically using microscopy.

Harvesting and culturing human nasal epithelial cells *in vitro*

Primary human nasal epithelial cells (HNECs) were harvested from nasal mucosa by gentle brushing in a method described by Ramezanpour et al. [285] Extracted cells were suspended in Bronchial Epithelial Growth Media (BEGM, CC-3170, Lonza, Walkersville, MD, USA), supplemented with 2% Ultroser G (Pall Corporation, Port Washington, NY, USA). The cell suspension was depleted of macrophages using anti-CD68 (Dako, Glostrup, Denmark) coated culture dishes, and HNECs were maintained with B-ALI™ growth medium (Lonza, Walkersville, USA) in collagen coated flasks (Thermo Scientific, Walthman, MA, USA) in a cell incubator at 37°C with 5% CO2.

Air liquid interface culture

HNECs were grown until 80% confluent then harvested for seeding onto collagen coated 6.5 mm permeable Transwell plates (BD Biosciences, San Jose, California, USA) at a density of 5×10^4 cells per well. Cells were maintained with B-ALI™ growth medium for 2-3 days in a cell incubator at 37°C with 5% CO2. Culture purity was confirmed morphologically using microscopy. On day 3 after seeding, the apical media was removed and the basal media replaced with B-ALI™ differentiation media, exposing the apical cell surface to the atmosphere. Human nasal epithelial cultures at air liquid interface (HNEC-ALI) were maintained for a minimum of 21 days prior to experimentation for development of tight junctions [286].

Antibiotics

An array of antibiotics relevant to intra and post-operative care in sinus surgery were selected as treatments (table 1). Two oxazolidinone antibiotics were also selected due to emerging evidence that this class may facilitate a reduction in surgical adhesions [287]. Treatments were formulated using high concentration stock in cell media, at

concentrations adapted from those reported in peer-reviewed literature when using common dosing regimens. For example, in a study of 59 patients receiving 1000mg amoxicillin twice daily, the median concentration of amoxicillin in nasal secretions was found to be 2.34 µg/mL [288], and so a dose of 2 µg/mL was selected for our study. Where reliable data on nasal concentrations was not found, serum or plasma concentrations were used. For example, a review of Clarithromycin pharmacodynamics found that average steady-state peak serum concentrations were 2.0 to 3.0 mg/L after 500 mg twice-daily dosing, and so a dose of 2.5 µg/mL was selected for our study [289]:

Antibiotic	Concentration	Concentration Reference	Procurement
Beta-lactams			
Amoxicillin	2 µg/mL	*Kment et. al.* [288]	Sigma
Amoxicillin/ Clavulanate (Augmentin)	1.75/0.25 µg/mL	*Kment et. al.* [288]	Sigma
Macrolides/ Lincosamides			
Erythromycin	2 µg/mL	*Kanoh and Rubin* [290]	Sigma
Clarithromycin	2.5 µg/mL	*Rodvold* [289]	Sigma
Azithromycin	1 µg/mL	*Kanoh and Rubin* [290]	Sigma
Clindamycin	5 µg/mL	*Kanoh and Rubin* [290]	Sigma
Roxithromycin	2.5 µg/mL	*Nilsen et. al.* [291]	Sigma
Oxazolidinones			
Linezolid	5 µg/mL	*Aytan et. al.* [287]	Sigma
Tedizolid	5 µg/mL	*Takeda et. al.* [292]	MedKoo
Miscellaneous (class)			
Mupirocin	250 µg/mL	*Kim and Kwon* [293]	Sigma
Ciprofloxacin (quinolone)	5 µg/mL	*Sachse et. al.* [294]	Sigma
Doxycycline (tetracycline)	5 µg/mL	*Welling et. al.* [295]	Sigma

Table 1: Antimicrobial agents and concentrations used as treatments during the wound healing assay. Sigma = Sigma-Aldrich (Merck), St Louis, USA, MedKoo = MedKoo Biosciences Inc., South Carolina, USA

To assess the effect of these antibiotics, cells were exposed to the above concentrations of antibiotic just prior to cell wounding.

A mitochondrially targeted antioxidant named mitoquinone (MedKoo Biosciences Inc., South Carolina, USA), was used as a negative control for intracellular ROS production. A dose of 2 µM was selected in line with similar studies [229, 296] .

Unscratched, untreated cells were used as a separate control, to establish the background ROS levels being produced by the cells in the experimental conditions.

Cytotoxicity studies

Primary human fibroblasts or HNECs were grown in phenol DMEM and BEGM (Lonza, Walkersville, USA) medium respectively. Cells were maintained in a fully humidified incubator with 5% CO2 at 37°C prior to cytotoxicity studies. Cells were exposed to antibiotics at plasma concentrations for 40 hours, followed by determination of lactate dehydrogenase (LDH) with a cytotoxicity detection kit (Promega, Madison, U.S.). Briefly, 50 µL of the supernatant from each well was mixed with 50 µL of LDH reagent and was incubated for 30 minutes in the dark at room temperature. The optical density (OD) was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) and compared across treatment and control groups. Negative control was medium only and positive control was an LDH standard included with the detection kit. Cell culture studies were performed as three independent experiments.

Wound healing (cell migration) assay

In the fibroblast wound closure assay, fibroblasts were seeded between passages 5 and 8 into 24 well plates and allowed to reach 80% confluence over 24 hours. A straight vertical scratch was made down through the fibroblasts and HNEC-ALI cell monolayers by using a 200 µl pipette tip. The media and cell debris was aspirated carefully and culture media with different concentrations of antibiotics or media only (negative control) added to each well for 40 hours. The wound closure (cell migration) was recorded using time-lapse

LSM700 confocal scanning laser microscopy (Zeiss Microscopy, Germany) in a temperature and CO2 controlled chamber. An image was recorded every 30 minutes for 4 hours, and then every 4 hours for a further 36 hours. Wound area in pixels was quantified manually for each image using ImageJ Software (v1.52a, National Institutes of Health, USA).

Evaluation of cellular reactive oxygen species activity

ROS were detected using a chemiluminescent probe: carboxylated 2′, 7′dichlorodihydrofluorescein diacetate (H2-DCFDA; Invitrogen Life Technologies, Carlsbad, CA, USA) that has been widely validated in the literature on oxidative stress [297]. This carboxylated analogue of H2-DCFDA increases intracellular retention of the molecule, making it suited to longer time-lapse studies [297].

Primary nasal fibroblast cells were cultured in phenol-red free DMEM with 10% FBS and seeded into black walled 96-well plates (Life Technologies, Australia) and incubated for 24 hr in a humidified incubator with 5% CO2 at 37°C. Fibroblasts and HNEC-ALI cultures were washed with PBS and 10 μM of H2-DCFDA was added for 1 hr, at 37°C in the dark. Cells were then washed with PBS and exposed to scratching injury by dragging a 200 μL pipette tip linearly on the confluent monolayers in the presence of an antibiotic. The fluorescence intensity was recorded using time-lapse LSM700 confocal scanning laser microscopy (Zeiss Microscopy, Germany) using filter range Ex/Em: 492/525 nm every 30 minutes for 4 hours, and every 4 hours for a further 36 hours.

Enzyme-Linked Immunosorbent Assay (ELISA)

To determine if the chosen concentrations of antibiotics provoked an inherent inflammatory response likely to invoke a hypertrophic scar or adhesion, an IL-6 ELISA was undertaken on the cell supernatants. Supernatants were collected from fibroblasts and HNECs after 40 hours of exposure to the antibiotics. Interleukin-6 (IL-6) protein levels were estimated with an ELISA kit using rat anti-human IL6 antibodies (BD Biosciences, New Jersey, USA), according to the manufacturer's instructions. All measurements were performed in duplicate using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). The tissue sample concentration was calculated from a standard curve and corrected for protein concentration. These values were compared between antibiotic treatment groups, as well as scratched and unscratched control groups.

Statistical analysis

Linear mixed modelling incorporating wound size, time points, antibiotics and cell line donor was used for statistical analysis of the wound closure and relative fluorescence across the treatment groups. ROS suppression and wound healing delay for each treatment was compared to scratched controls at each timepoint using a two-tailed Students t-test. Statistical analyses of the LDH and ELISA assays were carried out using ANOVA, followed by Tukey HSD post hoc test. These tests were performed using SPSS software (v25, International Business Machines, USA) and Microsoft Excel (v1905, Microsoft, USA). Statistical significance was defined as a P value of less than 0.05.

RESULTS

Effect of mitoquinone on the release of reactive oxygen species and cell migration of primary human nasal epithelial cells and primary nasal fibroblasts

Intracellular ROS production was measured using the fluorescent probe H2-DCFDA. To examine the influence of the treatments on sinonasal wound resealing *in vitro*, time course studies were performed during active wound closure.

Performing a linear scratch over the cell monolayers produced an immediate (within 5 minutes) significant increase in ROS production of approximately 35% compared to unscratched controls in both cell types (Figure 1A, F, C, H) ($p<0.05$). This activity was sustained beyond the initial injury and gradually increased to approximately 55% higher than unscratched controls by the time of wound closure, with the most intense activity being focused at the wound edge (Figure 1A, C). The increase in activity was mitigated by exposing the wound to the mitochondrial ROS inhibitor mitoquinone in both cell types, reducing ROS activity to background levels within 2 hours after application (Figure 1B, D, F, H). The inhibitory effect of mitoquinone on ROS production compared to control became significant earlier in fibroblasts (after 3 hours, Figure 1F) than HNEC (after 12 hours, Figure 1H).

In the absence of mitoquinone, the wound closed after 32-36 hours for both HNEC and fibroblasts. The presence of mitoquinone produced a marked increase in fibroblast transit times across the wound from the 12-hour mark onwards ($p<0.05$), leading them to close approximately 16 hours later than untreated samples (52 hours). This effect was not replicated in HNEC (Figure 1 E,G).

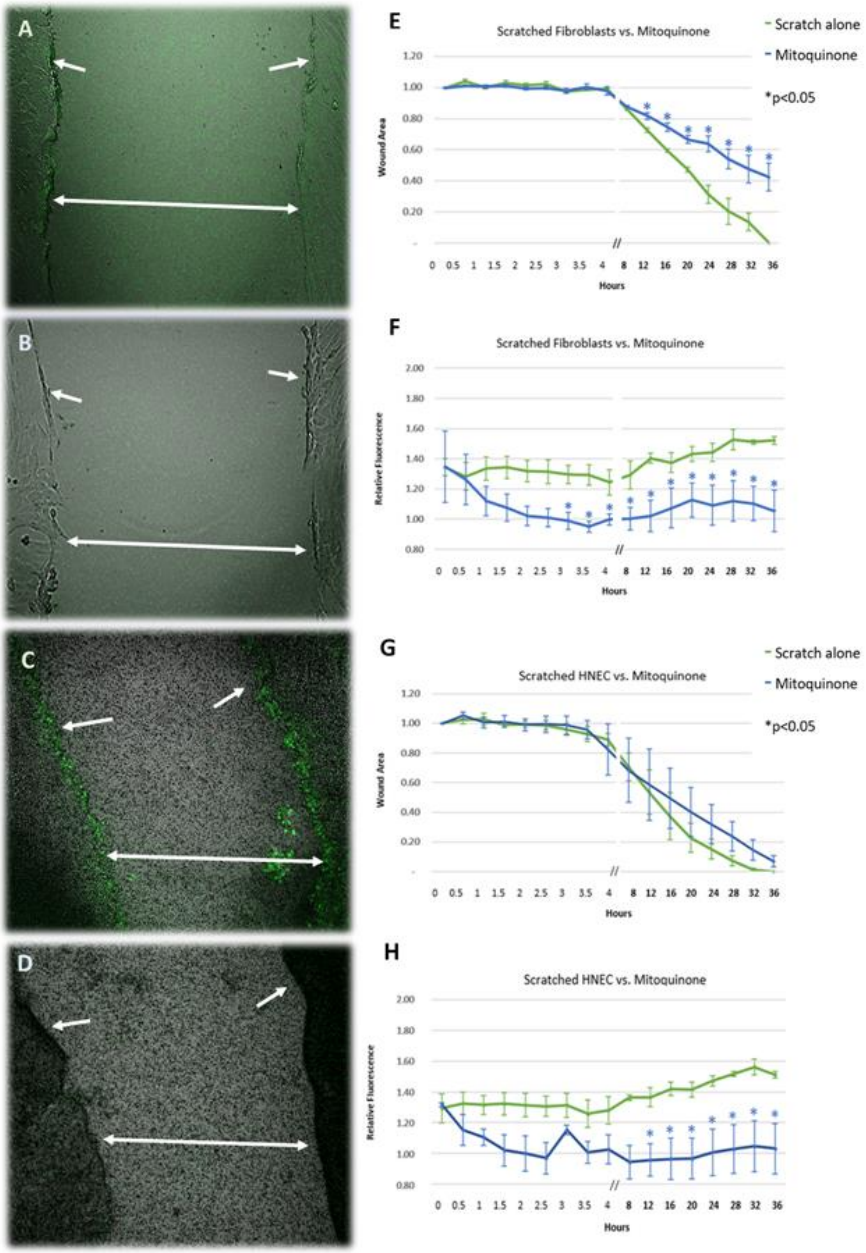

Figure 1: Effect of mitoquinone on the release of reactive oxygen species and cell migration of scratched primary human nasal fibroblasts (A, B, E, F) and primary human nasal epithelial cells (HNEC) (C, D, G, H). Representative images of standard mechanical wound of HNEC monolayer or fibroblasts with **(B, F)** and without **(A, C)** and with exposure to mitoquinone **(B, F)** with wound area **(E, G)** and relative fluorescence **(F, H)** measured over 36 hours in the presence (blue line) or absence (green line) of mitoquinone. Arrows indicate wound edge, double arrows span wound. Y-values in E, G represent mean proportion of original wound area remaining ± SEM (n=3). Y-values in F, H

represent fluorescence emitted by scratched monolayers above the levels in unscratched, untreated controls (normalised to 1) ± SEM (n=3). * p<0.05, T-test.

Effect of antibiotics on the release of reactive oxygen species and cell migration of primary human nasal epithelial cells and primary nasal fibroblasts

Amoxicillin, both as a sole agent and in combination with clavulanate, did not produce a significantly different pattern of ROS activity or cell transit times compared to control untreated samples (Fig. 2).

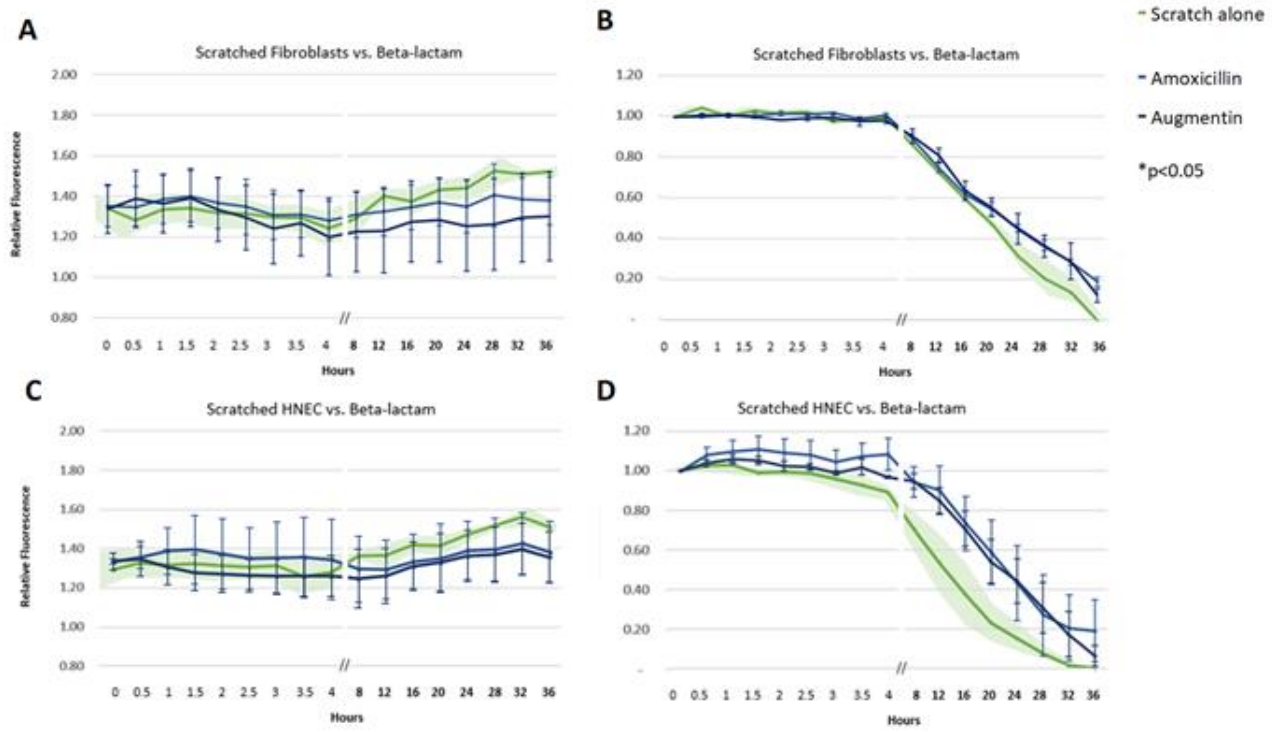

Figure 2: Effect of beta-lactam antibiotics on ROS production and cell migration of scratched cells. Mean relative fluorescence **(A, C)** and mean wound area **(B, D)** measured over 36 hours of scratched fibroblasts **(A, B)** and HNECs **(C, D)** treated with the beta-lactam antibiotics amoxicillin (blue line) or augmentin (black line) or scratched untreated control (green line and green shaded area). Y-values in **A, C** represent mean percentage of additional fluorescence emitted by scratched monolayers above unscratched, untreated controls ± SEM error bars (n=3). Y-values in **B, D** represent mean proportion of original wound area remaining ± SEM error bars (n=3).

The macrolide antibiotics (clarithromycin, erythromycin, azithromycin, roxithromycin) and lincosamides (clindamycin) had the widest variability of ROS response across fibroblasts and HNEC, with both cell types showing a trend towards reduction in ROS activity for these antibiotics (Fig. 3). Only clarithromycin showed a significant decrease in cell migration ($p<0.05$), from as early as 8 hours, in fibroblasts alone (Fig. 3 C).

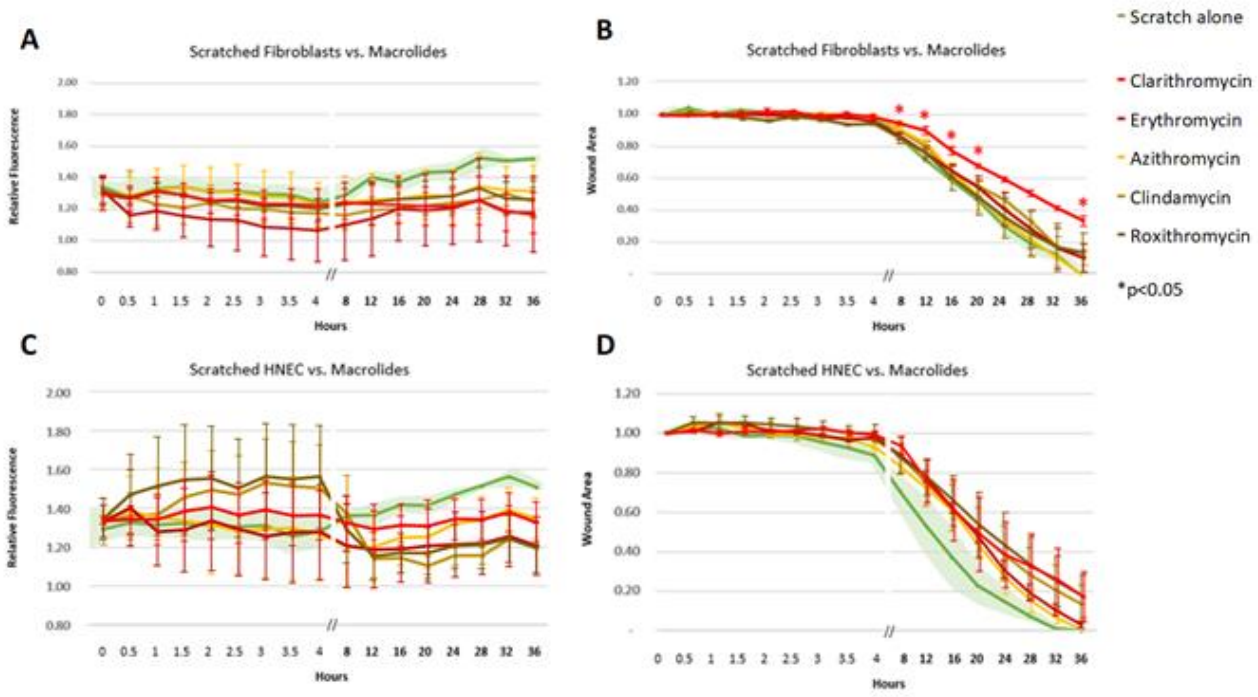

Figure 3: Effect of macrolide and lincosamide antibiotics on ROS production and cell migration of scratched cells. Mean relative fluorescence **(A, C)** and mean wound area **(C, B)** measured over 36 hours of scratched fibroblasts **(A, B)** and HNECs **(C, D)** treated with clarithromycin (red line), erythromycin (dark red line), azithromycin (yellow line), clindamycin (light brown), roxithromycin (dark brown line) or scratched untreated control (green line and green shaded area). Y-values in **A, C** represent mean percentage of additional fluorescence emitted by scratched monolayers above unscratched, untreated controls ± SEM error bars (n=3). Y-values in **B, D** represent mean proportion of original wound area remaining ± SEM error bars (n=3). * $p<0.05$, T-test.

Linezolid outperformed its oxazolidinone counterpart Tedizolid with a consistent strong reduction in ROS activity in scratched HNEC (significant from 20-hour time point onwards for linezolid and from the 24-hour time point onwards for tedizolid, Figure 4B) but not in scratched fibroblasts. In those cells, both antibiotics had a similar mean ROS activity which was significantly reduced compared to untreated scratched control only at the 36-hour time point for tedizolid (Fig. 4A). Both Linezolid (at 12 hours and onwards) and Tedizolid (at 16 hours and onwards) produced a consistent reduction in fibroblast transit times ($p<0.05$), however the effect of Linezolid was more pronounced (Fig. 4C). Both antibiotics did not affect HNEC transit times (Fig 4D).

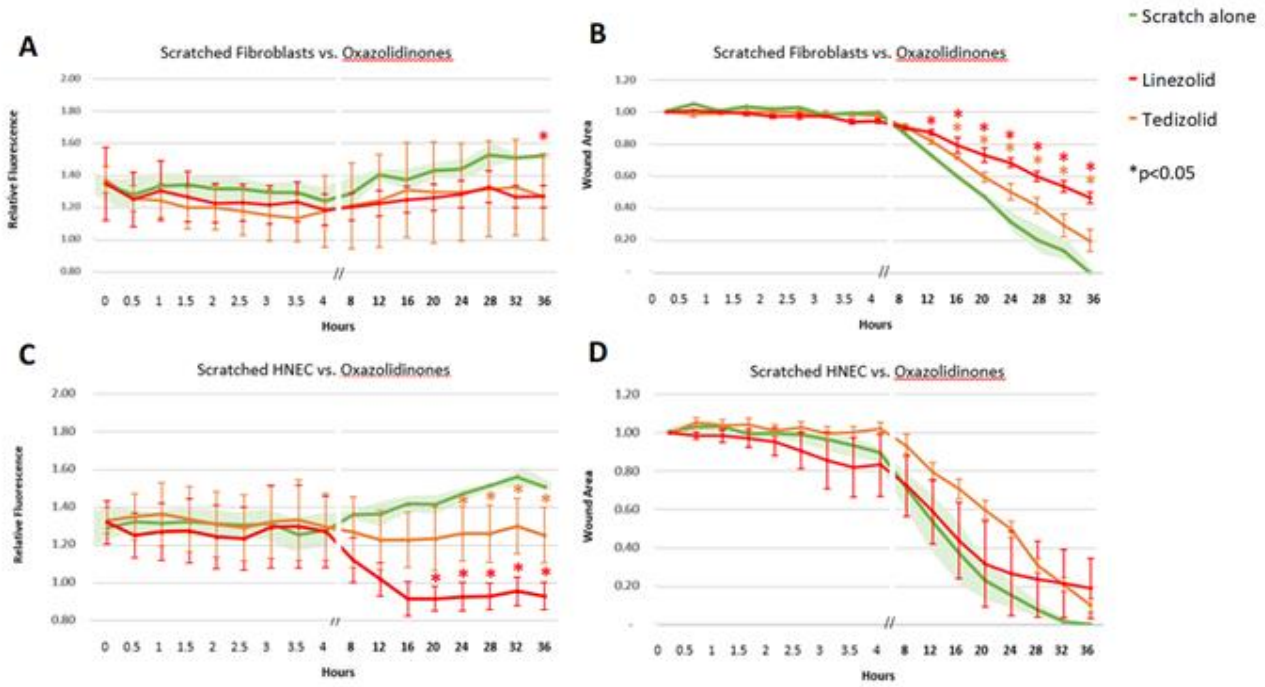

Figure 4: Effect of oxazolidinone antibiotics on ROS production and cell migration of scratched cells Mean relative fluorescence **(A, C)** and mean wound area **(B, D)** measured over 36 hours of scratched fibroblasts **(A, B)** and HNECs **(C, D)** treated with linezolid (red line), tedizolid (orange line) or scratched untreated control (green line and green shaded area). Y-values in **A, C** represent mean percentage of additional fluorescence emitted by scratched monolayers above unscratched, untreated controls ± SEM error bars (n=3). Y-values in **B, D** represent mean proportion of original wound area remaining ± SEM error bars (n=3). * $p<0.05$, T-test.

Of the remaining antibiotics included in the study, mupirocin, ciprofloxacin and doxycycline only achieved a trend towards fluorescence (ROS) reduction in the fibroblasts and HNEC (Figure 5 A, B). Mupirocin showed a significant reduction in fibroblast transit times from the 12-hour time point onwards (Fig. 5C, $p<0.05$). This slowing effect was not seen in HNEC for any of these three antibiotics (Fig. 5D).

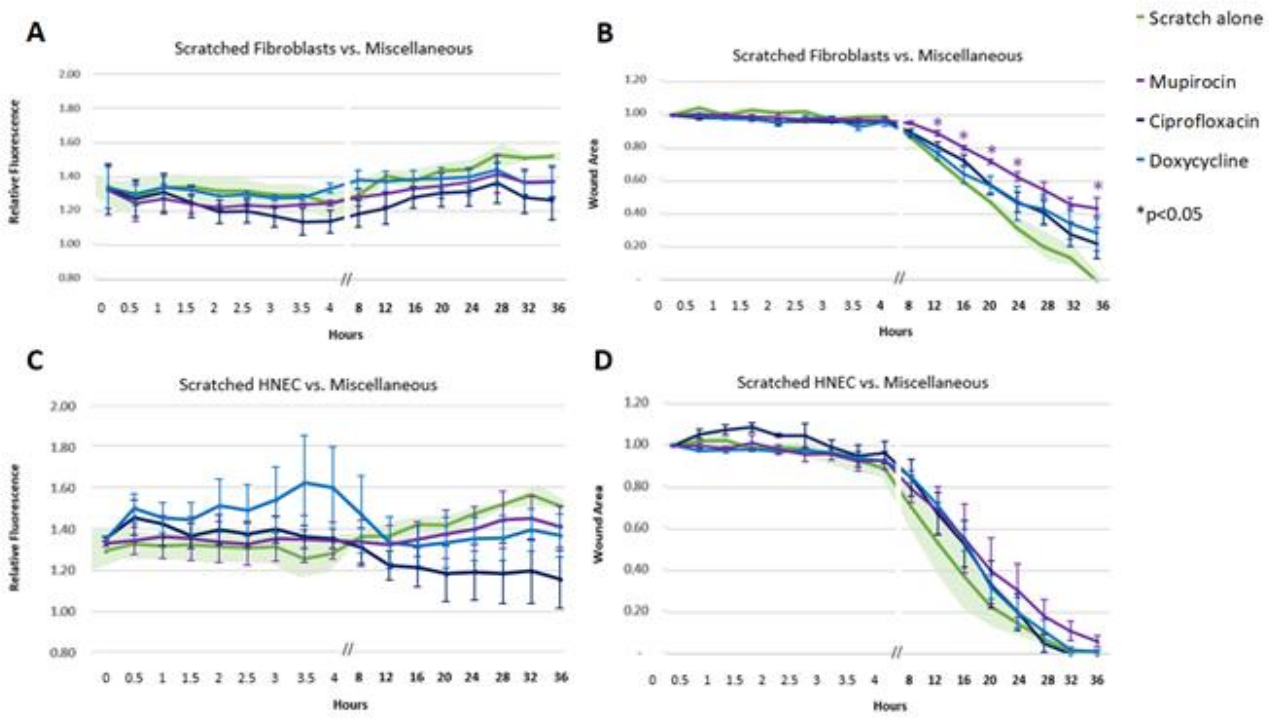

Figure 5: Effect of miscellaneous antibiotics on ROS production and cell migration of scratched cells. Mean relative fluorescence **(A, C)** or mean wound area **(B, D)** measured over 36 hours of scratched fibroblasts **(A, B)** and HNECs **(C, D)** treated with mupirocin (purple line), ciprofloxacin (dark blue line), doxycycline (light blue line) or scratched untreated control (green line and green shaded area). Y-values in **A, B** represent mean percentage of additional fluorescence emitted by scratched monolayers above unscratched, untreated controls ± SEM error bars (n=3). Y-values in **C, D** represent mean proportion of original wound area remaining ± SEM error bars (n=3). * $p<0.05$, T-test.

Relationship between ROS suppression and cell migration

Linear mixed modelling was used to detect a relationship between ROS suppression and cell migration rates. Mitoquinone and a majority of the antimicrobials (8 out of 10 tested) demonstrated a significant negative relationship between ROS levels and cell migration rate in fibroblasts. The exceptions were azithromycin and doxycycline, where no clear relationship was seen. The five treatments that significantly slowed fibroblast wound closure compared to untreated controls (Figs. 1-5) all showed a significant relationship with ROS suppression. These treatments included clarithromycin, mupirocin, the ROS inhibitor mitoquinone and both oxazolidinones, linezolid and tedizolid.

Fluorescence (ROS level) compared to untreated controls was generally lower for fibroblasts (Table 2) compared with HNEC (Table 3).

Fibroblasts			
Treatment	**ROS activity compared to controls**	**Migration rate**	**p <0.05**
Mitoquinone	-0.458	0.255	*
Linezolid	-0.307	0.110	*
Clindamycin	-0.300	0.172	*
Erythromycin	-0.277	0.102	*
Ciprofloxacin	-0.236	0.163	*
Tedizolid	-0.229	0.076	*
Roxithromycin	-0.227	0.252	*
Azithromycin	-0.224	0.132	
Augmentin	-0.201	0.129	*
Clarithromycin	-0.187	0.294	*
Mupirocin	-0.124	0.095	*
Doxycycline	-0.122	0.276	

Table 2: Linear mixed modelling estimates of fibroblast ROS inhibition and migration rate for each treatment, relative to untreated scratched controls (negative co-efficient for ROS activity corresponds with more inhibition). P-values indicate a significant relationship between the two for the given treatment (*, p<0.05). Values and treatments sorted by inhibition of ROS activity. Treatments in bold also showed a significant reduction in wound closure time compared to untreated wounds.

In the HNEC, azithromycin, augmentin, clarithromycin and doxycycline did not demonstrate a clear relationship between levels of ROS suppression and cell migration rate. Mitoquinone, the only treatment that significantly slowed HNEC wound closure compared to untreated controls, showed a significant relationship with ROS suppression ($p<0.05$).

Human Nasal Epithelial Cells			
Treatment	**ROS activity compared to controls**	**Migration rate**	**p <0.05**
Mitoquinone	-0.376	0.072	*
Linezolid	-0.345	0.091	*
Clindamycin	-0.216	0.168	*
Erythromycin	-0.171	0.128	*
Ciprofloxacin	-0.152	0.056	*
Tedizolid	-0.134	0.205	*
Roxithromycin	-0.127	0.196	*
Azithromycin	-0.112	0.090	
Augmentin	-0.087	0.185	
Clarithromycin	-0.071	0.183	
Mupirocin	-0.030	0.094	*
Doxycycline	0.006	0.064	

Table 3: Linear mixed modelling estimates of HNEC ROS inhibition and migration time for each treatment, relative to untreated scratched controls. P-values indicate a significant relationship between the two for the given treatment (*, $p<0.05$). Values and treatments sorted by inhibition of ROS activity. Treatments in bold also showed a significant reduction in wound closure time compared to untreated wounds.

Cell Migration Across Cell Types

The majority of treatments slowed fibroblast migration more than they slowed HNEC migration. Amoxicillin +/- clavulanate was a notable exception, where HNEC migration was slower than fibroblasts, along with tedizolid and erythromycin. The treatments that produced the biggest differentials towards slowing fibroblasts compared to HNEC were doxycycline, clarithromycin, ciprofloxacin and the ROS inhibitor mitoquinone (Table 4).

Treatment	Fibroblasts	HNEC	Differential
Doxycycline	0.276	0.064	-0.212
Mitoquinone	0.255	0.072	-0.183
Clarithromycin	0.294	0.183	-0.111
Ciprofloxacin	0.163	0.056	-0.107
Roxithromycin	0.252	0.196	-0.056
Azithromycin	0.132	0.09	-0.042
Linezolid	0.110	0.091	-0.019
Clindamycin	0.172	0.168	-0.004
Mupirocin	0.095	0.094	-0.001
Erythromycin	0.102	0.128	0.026
Augmentin	0.129	0.185	0.056
Amoxicillin	0.167	0.225	0.058
Tedizolid	0.076	0.205	0.129

Table 4: Linear mixed modelling estimates of wound closure for each treatment, relative to untreated controls (higher co-efficient corresponds with slower wound healing). Negative differential represents tendency for slowing fibroblasts relative to HNEC, positive differential represents tendency for slowing HNEC relative to fibroblasts.

DISCUSSION

Whilst many bactericidal antibiotics have been shown to provoke ROS activity in undamaged cells [194-196], their effect in the post-operative setting, where they are often used empirically to prevent infection, has been hitherto unknown. Our study elucidates the effect of a wide range of commonly used antibiotics on ROS activity in damaged fibroblast and HNEC cell layers, at concentrations that reflect postoperative dosages. In this setting, all antibiotics produced either a suppressive or non-stimulatory effect on the ROS production occurring in response to the mechanical wound. This effect was always significant where reduced cell migration was observed.

Amoxicillin/ clavulanate, an agent favored in rhinology for its activity against a majority of common nasal pathogens [283, 298, 299], did not produce a significant reduction in ROS activity across either of the cell types. It also did not create a differential between fibroblast migration and epithelialization, indicating that it has little intrinsic benefit in preventing sinonasal adhesions after surgery.

It has been well demonstrated that the critical time period to block postoperative adhesions is the first few days after the initial injury. The extent of adhesion formation is multifactorial, and depends significantly on the level of inflammation, ROS production and fibroblast migration during that time [300]. Fibroblasts are responsible for collagen synthesis, and a decrease in collagen synthesis and fibroblast cell migration results in slower, more measured wound repair that is less likely to result in an adhesion [301, 302]. Our data implies that some, but not all, antibiotics might be beneficial in preventing adhesions after nasal surgery as they limit fibroblast cell adhesions without negatively affecting the re-epithelialization.

Clarithromycin emerged as an antimicrobial that was able to consistently slow fibroblast transit times compared with epithelial cells, and there was a demonstrable link between this effect and the degree of ROS inhibition the treatment was able to achieve. Macrolides have long been shown to suppress oxidative burst in human immune cells [290, 303], and

the present study links this effect to beneficial cell migration profiles in the *in vitro* setting. Further studies are required to evaluate whether this finding translates into reduced adhesion formation after sinus surgery.

Doxycycline also achieved a beneficial differential between fibroblast and epithelial cell migration, though this was not attributable to ROS inhibition. The positive effect of tetracyclines on chronic wounds have previously been credited to their immunomodulatory and anti-inflammatory actions, specifically through the inhibition of matrix metalloproteinases [304], and our study does not suggest ROS play an additional role. Rather, our findings reinforce the notion that cell migration and wound closure is multifactorial and that ROS play an important, though not exclusive, role here. This effect is more pronounced in the fibroblast cell type when compared with HNEC.

Though included primarily as a control for ROS inhibition in this experiment and selected for its mechanistic specificity at the mitochondria (where antibiotics are also theorized to exert their ROS modulatory effects [196]) mitoquinone had the most beneficial effect on cellular migration profiles across the wound and warrants more focused investigation as a potential anti-adhesion product in the postoperative setting. Whilst antioxidants are not currently used widely in rhinology for this purpose, they have previously shown promise in animal models of nasal wound healing [230, 247, 305] and merit further investigation in human trials.

As expected, the antibiotic concentrations derived from the literature to mimic typical postoperative plasma concentrations did not alter LDH release in any of the cell lines. This adds to the translational significance of the present study, as the reduction in wound migration cannot be attributed to cell damage and reflects the physiological response of a postoperative patient receiving antibiotics in the days following surgery. These concentrations also did not up-regulate production of IL-6 in the cells. In an *in vitro* wound model of hypertrophic scar fibroblasts, a microarray analysis indicated the interleukin 6 (IL-6) signaling pathway to be the main pathway involved in the early response to injury in

those cells [306]. Moreover IL-6, along with other pro-inflammatory factors such as interleukin (IL)-1α, IL-1β and tumor necrosis factor-α are up regulated in hypertrophic scar tissues [286, 307].

Certain limitations must be considered for the present study. The wide variety of antimicrobial agents included in the analysis limited the resources available for repeat experimentation and may explain the occasionally high variability of responses. Care was taken to repeat each experiment in triplicate across multiple tissue donors as a minimum, to allow for calculation of the standard error of the mean. The value of this approach is that our study now provides a breadth of data that will direct more focused analysis of the agents with the most beneficial effects on wound healing.

The *in vitro* nature of the wound healing experiment limits the clinical application of the data in its current form, particularly as the key influence of the immune system is difficult to factor in. The inflammatory response of the immune system is instrumental to supplying growth factor and cytokine signals that orchestrate the cell and tissue movements necessary for repair [300]. Their absence in this model is likely to have had a significant impact on the cellular behavior observed. Nevertheless, the present study is a necessary step in the move towards *in vivo* experimentation utilizing models with a functioning immune system. It also delineates the inherent properties of the cells from any observed effects *in vivo*.

CONCLUSION

In this translational model of postoperative nasal wound healing, ROS suppression from antioxidant or antibiotic exposure was associated with a slowed sinonasal cell migration across newly formed wounds *in vitro*. This effect was observed more reliable and profoundly in fibroblasts compared with epithelial cells. Many of the antibiotics used in rhinological practice created more favorable wound healing profiles through the

preferential inhibition of fibroblast migration over epithelial cells, with amoxicillin/clavulanate being a notable exception. These findings strengthen the notion that ROS modulation is an important mechanism for supporting optimal wound healing post-operatively.

Statement of Authorship

Title of Paper	*In Vitro* Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Treating *Staphylococcus aureus* Biofilms
Publication Status	Prepared for Submission
Publication Details	Withheld Under Embargo

Principal Author

Name of Principal Author (Candidate)	Dr Michael Gouzos		
Contribution to the Paper	Project design, experimental work, data collection and analysis, manuscript preparation		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/12/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Clare Cooksley		
Contribution to the Paper	Project design, experimental support, data analysis, manuscript preparation		
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In Vitro Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Treating *Staphylococcus aureus* Biofilms

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Abstract

Background Biofilms are an important entity in severe upper respiratory tract infections that drive bacterial persistence and chronic inflammation. Biofilms comprising staphylococcal species are frequently identified in patients with recalcitrant infection. Systemic antibiotics are often insufficient to treat these biofilms, provoking the need for alternate treatments. Antioxidants are one class of therapies that have shown promise in treating and preventing biofilms. The present study aims to evaluate both the *in vitro* safety and antimicrobial efficacy of treating established biofilms with two separate mitochondrially-targeted antioxidants (MTAs).

Methods An effective range of the MTAs mitoquinone and visomitin were tested against both *S*. *aureus* and MRSA, by measuring optical density, colony forming units (CFUs), alamar blue assays, crystal violet assays and live-dead staining with confocal laser scanning microscopy. MTAs were then tested on human cells to exclude cytotoxicity, barrier disruption and cilia dysfunction. Combination therapy with common antibiotics was also analysed to assess potential synergy.

Results Both MTAs showed a significant ($p<0.05$) dose-dependent growth suppression against both planktonic cells and biofilms of *S. aureus* ATCC 25923 and two MRSA clinical isolates. Notably, 5μM mitoquinone suppressed visible growth of all three strains, killed an average of 69% of established biofilm and reduced their metabolism by 51% ($p<0.05$), whilst not provoking toxicity or dysfunction in human cells ($p<0.05$). It also improved the activity of augmentin, doxycycline and clarithromycin ($p<0.05$) against all strains of planktonic and biofilm *S. aureus* and MRSA.

Conclusions MTAs contain an antioxidant base that targets mitochondrial ROS, leading to a more favorable inflammatory profile, as well as an effective antimicrobial moiety against the biofilms of at least one significant pathogen. It may also be combined with existing antimicrobials to antagonise biofilms even more effectively.

INTRODUCTION

Staphylococcus aureus infection presents several challenges within the human body, due to several mechanisms that the bacteria may employ to avoid eradication. These include a combination of transient quiescence, to avoid stimulation of an immune response, and the production of defensive polymeric substances, designed to veil and physically protect themselves from exogenous threats. In this way, many bacteria like *Staphylococcus* may evade medical treatments that were originally conceived under the paradigm of free floating, 'planktonic' bacteria, freely accessible to antimicrobial therapy. Modern paradigm shifts in microbiology have demonstrated that the vast majority (>99%) of bacteria are not planktonic, instead forming clusters of cells encased in a polymeric matrix, collectively termed a biofilm. [15, 16] These biofilms not only cause failure of traditional medical treatments but increase the risk of recurrent infections, by providing a reservoir of bacteria within the body, ready to emerge when treatments have ceased or the immune response has settled. [12, 13, 17]

Different biofilm species are associated with different disease phenotypes, particularly in the upper respiratory tract. *Haemophilus influenzae* biofilms are typically found in patients with milder disease, whereas *S. aureus* is associated with a more severe, recalcitrant pattern [18]. *S. aureus* is a particularly problematic pathogen because it also has the potential to cause intracellular infections, which can evade host immunity in a similar manner to biofilms. [12, 13]

Biofilm formation may be involved in many infections, including ventilator-associated pneumonia, cystic fibrosis, bronchiectasis, bronchitis, and upper respiratory airway infections. [17] Given the severe complications caused by these recalcitrant infections, several non-antibiotic compounds have been investigated *in vitro* as potential biofilm treatments. Antioxidants are one such example. There is level I evidence that non-targeted antioxidants such as N-acetyl cysteine (NAC) add efficacy to antibiotic treatment of a variety of significant nosocomial pathogens, including *S. aureus* [308], as

well as directly reducing biofilm biomass. [309] One of the drawbacks of this therapy is the high dosage required to see a therapeutic effect - as high as 80mg/mL in some studies. [310, 311]

One method to overcome this drawback has been to develop antioxidants designed to accumulate deep within the metabolic 'engine room' of the cell, the mitochondrial matrix. The most extensively studied of these mitochondrially-targeted antioxidants (MTAs) is mitoquinone, which contains an antioxidant ubiquinone moiety (co-enzyme Q_{10}) covalently attached to a lipophilic triphenylphosphonium cation. [247] This property enables mitoquinone to accumulate more efficiently within the mitochondria than standard antioxidants, where it reduces cellular levels of reactive oxygen species (ROS). [312] A similar MTA named visomitin (SkQ1) has shown promise in the treatment of inflammation associated with ocular surface diseases such as dry eye disease and corneal wounds, and is already being used therapeutically for this purpose. [313]

Of considerable interest, MTAs have also been demonstrated to have antimicrobial activity against a range of planktonic bacteria. [255, 314] Their efficacy against biofilms, however, is crucially hitherto unknown. The present study aims to explore the *in vitro* safety and efficacy of MTAs for antagonizing *S. aureus* and MRSA biofilms, both alone and in combination with existing antimicrobials.

MATERIALS & METHODS

Study population

This study was performed in accordance with guidelines approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital and the University of Adelaide (reference HREC/15/TQEH/132). All patients that donated sinonasal cells and/or bacteria gave written, informed consent and all samples obtained were anonymised and coded before use. All patients recruited to the study were undergoing endoscopic sinus surgery for chronic rhinosinusitis (CRS), diagnosed using well-established criteria [3, 4]. Exclusion criteria included active smoking, age less than 18 years, pregnancy, systemic immunosuppressive disease and underlying malignancy.

Preparation of treatments

Mitoquinone mesylate (MedKoo Biosciences Inc., South Carolina, USA) was prepared as high concentration stock (3mg/mL) in 1:1 ethanol: milliQ water as per the manufacturer's instructions. All treatments (1µM - 20µM) were formulated using <1% dilutions of this stock in cell media or broth. 1:1 ethanol: milliQ dilutions were also assessed as a vehicle control.

Key experiments were repeated using another MTA -Visomitin bromide (MedKoo Biosciences Inc., South Carolina, USA). This was prepared as high concentration stock (3mg/mL) in 1:1 ethanol: milliQ water as per the manufacturer's instructions, in an identical fashion to mitoquinone.

Antibiotics used for combination treatments were the beta-lactam amoxicillin with clavulanate (875 to 125 ratio), the tetracycline doxycycline, the macrolide clarithromycin and mupirocin (all acquired from Sigma Aldrich, Steinheim, Germany). Antimicrobial synergism with MTA was calculated using a checkerboard assay and calculating the fractional inhibitory concentration (FIC). [315]

Bacteria

S. aureus used in bacterial studies was propagated from glycerol stocks of both a reference strain known to produce biofilms (ATCC 25923, American Type Culture Collection, Manassas, USA) and two clinical isolates (CIs) of methicillin resistant *S. aureus* (MRSA) sourced from 2 patients with CRS undergoing sinus surgery (see 'Study Population').

Harvesting and culturing primary human nasal fibroblasts *in vitro*

Sinonasal tissue was biopsied from paranasal sinus mucosa and transferred to a culture plate with Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, UK) supplemented with L-glutamine, fetal bovine serum (FBS, Sigma, St Louis, USA) and penicillin streptomycin (Gibco, Life Technologies, New York, USA). Every 2-3 days, the tissue was washed gently with phosphate-buffered saline (PBS) and medium was replaced with fresh medium until fibroblasts became confluent after approximately 2 weeks. Confluent fibroblasts were washed with PBS, trypsinized and collected using centrifugation. Centrifuged pellets were resuspended in PBS along with Dynabeads Epithelial Enrich (Invitrogen, New York, USA). The tube was wrapped in parafilm and placed on a rotor mixer for 20 minutes at room temperature. Supernatant containing fibroblasts was then transferred to a tissue culture flask (Nunc, Roskilde, Denmark).

Harvesting and air-liquid interface culturing of human nasal epithelial cells *in vitro*

Primary human nasal epithelial cells (HNECs) were harvested from nasal mucosa using an established protocol. [285] Cells were suspended in PneumaCult™-Ex Plus Medium (STEMCELL Technologies Australia, Tullamarine, Australia) and depleted of macrophages using anti-CD68 (Dako, Glostrup, Denmark) before being cultured with Ex Plus Medium in collagen coated flasks at 37°C with 5% CO_2. HNECs were grown until confluent and seeded onto collagen coated 6.5 mm permeable Transwell plates (Corning Incorporated, Corning, USA) at a density of 5×10^4 cells per well. Cells were maintained

with Ex Plus medium for 2 days in a cell incubator at 37°C with 5% CO_2. On day 3 after seeding, the apical media was removed and the basal media replaced with PneumaCult™-ALI differentiation media (STEMCELL Technologies Australia, Tullamarine, Australia), exposing the apical cell surface to the atmosphere. HNEC at air liquid interface (HNEC-ALI) were maintained for a minimum of 21 days prior to experimentation for development of tight junctions and ciliation. [286]

Safety Studies

Cytotoxicity studies

Primary human fibroblasts or HNECs were maintained in a fully humidified incubator with 5% CO_2 at 37°C prior to cytotoxicity studies. Cells exposed to graded micromolar concentrations of MTA or control (medium only) for 48 hours, followed by determination of lactate dehydrogenase (LDH) with a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA). Briefly, 50 µL of the supernatant from each well was mixed with 50 µL of assay reagent and incubated for 30 minutes in the dark at room temperature. After addition of stop solution, the optical density (OD) was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) and compared across treatment and control groups. Medium alone was used as the negative control and an LDH standard included with the detection kit was tested for quality assurance. Cell viability was determined by comparing LDH release of the untreated control with that of treatment groups.

Cilia beat frequency

Cilia Beat Frequency (CBF) of HNEC-ALI cultures was assessed using a 20× objective, and 1.5x magnification on an inverted microscope (Olympus IX70, Tokyo, Japan). Video was recorded using a Model Basler acA645-100µm USB3 camera (Basler AG, Ahrensburg, Germany) at 100 frames per second at a resolution of 640 × 480 pixels. The recorded video samples were analyzed using the Sisson-Ammons Video Analysis (SAVA)

system. All measurements were taken at room temperature. A baseline CBF was taken prior to addition of the experimental conditions. Initially HNEC-ALI cultures were washed with 100 µL of PBS then 40 µL of PBS was added to the apical compartment. Baseline measurements were taken from 2 separate regions from each culture. The apical PBS was removed and either the control media, vehicle only control, or mitoquinone treatment was added. Sequential readings were taken every 5 minutes for the first 20 minutes to assess for any early stimulation/slowing of the CBF. Readings were also taken at 6 and 24 hours. Results are expressed as the mean CBF (Hz) and also as a percentile change when compared to baseline.

Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) of HNEC-ALI cultures was measured using an EVOM2 epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL) and results were recorded as a differential in ohms between the apical and basal chambers. 100 µL of PBS (1x) medium was added to the apical chamber of ALI cultures to form an electrical circuit across the cell monolayer and into the basal chamber. Cultures were maintained at 37°C during the measurement period using a heating platform. The corresponding mitoquinone treatment or control (ALI medium for the negative control and 2% Triton ×100 for the positive control) was added to the bottom chamber of each well, and TEER measurements were obtained hourly for 4 hours, then 4-hourly until 16 hours.

FITC-Dextran membrane integrity

After completion of the required treatments on HNEC-ALI cultures, FITC-dextran (Sigma Aldrich, Steinheim, Germany) was added to a final concentration of 0.3 mg/ml in 100 µL PneumaCult-ALI medium to the apical chamber of the transwell plate. Cells were then incubated 37 °C, protected from light, for 2 hours. 40 µL of the media from the bottom (basal) chamber was then transferred to a 96 well plate and its fluorescence read on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) using wavelengths 485 nm and 520 nm for excitation and emission respectively.

Anti-bacterial and anti-biofilm efficacy studies

Minimal Inhibitory Concentrations (MIC) assay

MIC of the test compounds against three bacterial strains was ascertained using conventionally described methods. [316, 317] Briefly, bacterial colonies were grown overnight on Mueller-Hinton agar and then transferred to Mueller-Hinton broth (MHB) the following day to create a 0.5 McFarland (MFU) bacterial inoculum. This inoculum was diluted 1:100 in MHB and divided into four separate treatment arms: inoculum only or

inoculum with either 1µM MTA, 2µM MTA or 5µM MTA. 50µL of inoculum +/- MTA was added to each treatment well.

For antimicrobial synergism assays (i.e. checkerboard assays) the inoculum was then treated with 50µl serial dilutions of four antimicrobial agents used commonly for the treatment of staphylococcal infections: amoxicillin and clavulanate, doxycycline, clarithromycin and mupirocin.

Treatments were incubated for 16hrs at 37ºC and repeated in triplicate for each of the three strains. Wells with the bacterial inoculum in broth (i.e. 100% bacterial growth) were used as negative control and wells with pure broth (i.e. 0% bacterial growth) were used as a baseline reference and sterility check. Inoculum strength was confirmed by diluting and plating maximal growth control on Mueller-Hinton agar and comparing the number of colony-forming units (CFU) formed per mL with the required range. [316, 317]

Antimicrobial synergism was interrogated using an established ‘’checkerboard’’ assay [318], followed by calculating the fractional inhibitory concentrations (FIC) of mitoquinone with four different antibiotics, whereby:

ΣFIC = FIC (mitoquinone) + FIC (antibiotic)

and

FIC (x) = MIC of combination / MIC(x).

ΣFIC ≤ 0.5 was taken as synergism, ΣFIC 0.5 - 4 was taken as indifference and ΣFIC > 4 was taken as antagonism. [318]

CFU assay

MTA treatments were set up as per the MIC Assay. After 16 hours exposure the treated bacteria were serially diluted and plated back onto Mueller-Hinton agar in triplicate. These plates were then incubated for a further 24 hours and read the following day. Numbers of colonies were counted and averaged across the three replicates for each

treatment. The number of CFU/mL was then derived for each of the three doses of MTA and the control (broth only).

In vivo efficacy

To assess the efficacy of mitoquinone against *S. aureus*, an assay using Caenorhabditis elegans (*C. elegans*) AU37 (glp-4; sek-1) was performed. Briefly, to synchronize the life cycle of the worm population, they were collected from Nematode Growth Medium (NGM) agar plates an E. *coli* OP50 lawn at 16 °C by washing with 0.9% saline. Then, a mixture of sodium hydroxide and 5% sodium hypochlorite was used to lyse the worms so that the eggs could subsequently be harvested. Synchronized nematodes (L4 stage) were collected with medium containing 95% M9 buffer, 5% BHI supplemented with 10 µg/ml cholesterol (OGM medium) and seeded in a 96-well plate to approximately 20 worms per well. Then they were treated with 5 µM mitoquinone, OGM medium and S. *aureus* ATCC 25923 suspension. Worms in OGM medium and bacterial suspension/OGM medium were considered negative and infection control, respectively. CFU per worm was measured after 24 hours incubation at 20 °C. Nematodes were collected and rinsed with M9 buffer supplemented with 1 mM sodium azide. They were then washed with PBS before being mechanically disrupted, before being vortexed with 1.0 mm silicon carbide beads (Biospec Products, Oklahoma, USA) for 10 minutes. Finally, CFU were counted by spreading dilutions of the supernatant on 7% NaCl agar plates and represented as a CFU/worm value.

Biofilm formation

Single colonies of *S*. *aureus* were immersed in 0.9% saline, adjusted to 1.0 ± 0.1 MFU (3 × 10^8 colony forming units/mL), and diluted 1:15 in nutrient broth (Oxoid Ltd., Basingstoke, United Kingdom). Then 150 µL of the diluted bacterial suspension was added to each well of a black 96-well microtiter plate (Costar R; Corning Incorporated, Corning, USA) and incubated in the dark at 37°C for 48 hours on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Australia) at 70 rpm.

Biofilm treatment

Biofilms were washed with PBS to remove planktonic cells, followed by exposure to either (1) serial dilutions of an antibiotic alone, (2) three micromolar concentrations of MTA (1μM, 2μM and 5μM), (3) combination of (1) and (2). After 12 hours of treatment exposure, with incubation in the dark at 37°C on a rotating platform, biofilms were washed with PBS to remove the excess treatment prior to being interrogated. Wells with the bacterial inoculum in broth (i.e., 100% bacterial growth) were used as negative control and wells with pure broth (i.e., 0% bacterial growth) were used as a baseline reference. All experiments were performed on all three strains of bacteria in triplicate.

Biofilm assessment

Biofilm activity was assessed in microtiter plates using the Alamar Blue viability assay, as conventionally described. [319] After drying, 200 µL of a freshly prepared 10% Alamar Blue dilution (Life Technologies, Scoresby, Australia) in nutrient broth was added to each well. Plates were incubated protected from light at 37°C on a rotating platform for up to 3 hours. The fluorescence was measured hourly on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at λexcitation = 530 nm/λemission = 590 nm. Maximum intensities were typically reached after 3 hours incubation and used for quantification. Results were expressed as percentage of maximal biofilm metabolism (i.e. a given treatment's fluorescence intensity as a percentage of the intensity in the maximum growth control at the same time point).

Biofilm viability was assessed in microtiter plates using the Crystal Violet assay, as conventionally described. [319] After drying, 180 µL of a freshly prepared 0.1% Crystal Violet dilution (Life Technologies, Scoresby, Australia) in water was added to each well and incubated for 15 minutes to stain the specimens. Plates were then rinsed 3 times with 200 µL sterile MilliQ water and dried. 180 µL per well of 30% acetic acid was used to solubilise the crystal violet into a homogenous solution, which was then read. The absorbance was measured once on an iMark™ Microplate Absorbance Reader (Bio-Rad, California, USA) at 595 nm. Results were expressed as percentage of viable biofilm (i.e. amount of signal absorbance as a percentage of the absorbance of the maximum growth control).

Biofilms were also qualitatively assessed using BacLight staining and confocal microscopy. Biofilms were grown under the same conditions above in an 8-well Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Fisher Scientific, Waltham, USA), washed twice in PBS to remove excess planktonic bacteria, and fixed using 5% glutaraldehyde fixative (Sigma Aldrich, Steinheim, Germany) for 45 minutes at 21^0C. Biofilms were then washed again with PBS to remove excess fixative. Dye was then

added to the wells (1 mL of MilliQ water containing 1.5 ul each of both LIVE/DEAD BacLight stains SYTO9 and propidium iodide) (Invitrogen Molecular Probes, Mulgrave, Australia) and slides were then incubated in the dark at 21^0C for 15 minutes. Biofilms were given a final wash with PBS to remove excess dye, and observed in a Zeiss LSM700 confocal LASER microscope (Zeiss Microscopy, Germany) at 10x magnification for LIVE/DEAD BacLight staining using two channels of a split beam: λexcitation = 488 nm/λemission = 518 nm (LIVE, represented as green) and λexcitation = 555 nm/λemission = 585 nm (DEAD, represented as red). A 14-slice (93µm total) z-stack was also taken to gain an appreciation of the 3D structure of the biofilm, and these were transposed upon one another into a representative 2D image using Zen microscope software (Zeiss Microscopy, Germany).

Statistical analysis

For experiments utilizing measures over multiple timepoints (CBF, TEER), a repeated-measures ANOVA and Dunnett's *post hoc* test was used for statistical analysis to compare treatments with controls.

For experiments comparing treatment groups at a final endpoint (bacterial studies, LDH assay etc.), groups were compared to controls using a Student's t-test. Data are presented as the mean ± SEM unless otherwise stated.

These tests were performed using SPSS software (IBM, U.S.A., v26). Statistical significance was defined as a p-value of less than 0.05.

RESULTS

Anti-bacterial and anti-biofilm efficacy studies

Mitochondrially-targeted antioxidants (MTAs) reduce the growth of planktonic *S. aureus*

The antimicrobial properties of mitoquinone and visomitin were tested on *S. aureus* ATCC 25923 and on 2 MRSA clinical isolates harvested from the nasal cavity of two CRS patients.

16hr of mitoquinone treatment resulted in a significant dose-dependent reduction in planktonic *S. aureus* CFU/milliliter. At 1μM it produced a significant reduction in only 1 of 3 strains (77% reduction of MRSA5743 compared to positive control, $p<0.05$). 2μM and 5μM mitoquinone significantly reduced planktonic growth of all strains ($p<0.05$).

Visomitin had similar activity after 16hrs of exposure. 1μM produced a significant reduction in both *S. aureus* ATCC 25923 and MRSA5743, the same CI that was susceptible to 1μM mitoquinone. 2μM and 5μM both produced a significant reduction in CFU/mL for all strains ($p<0.05$):

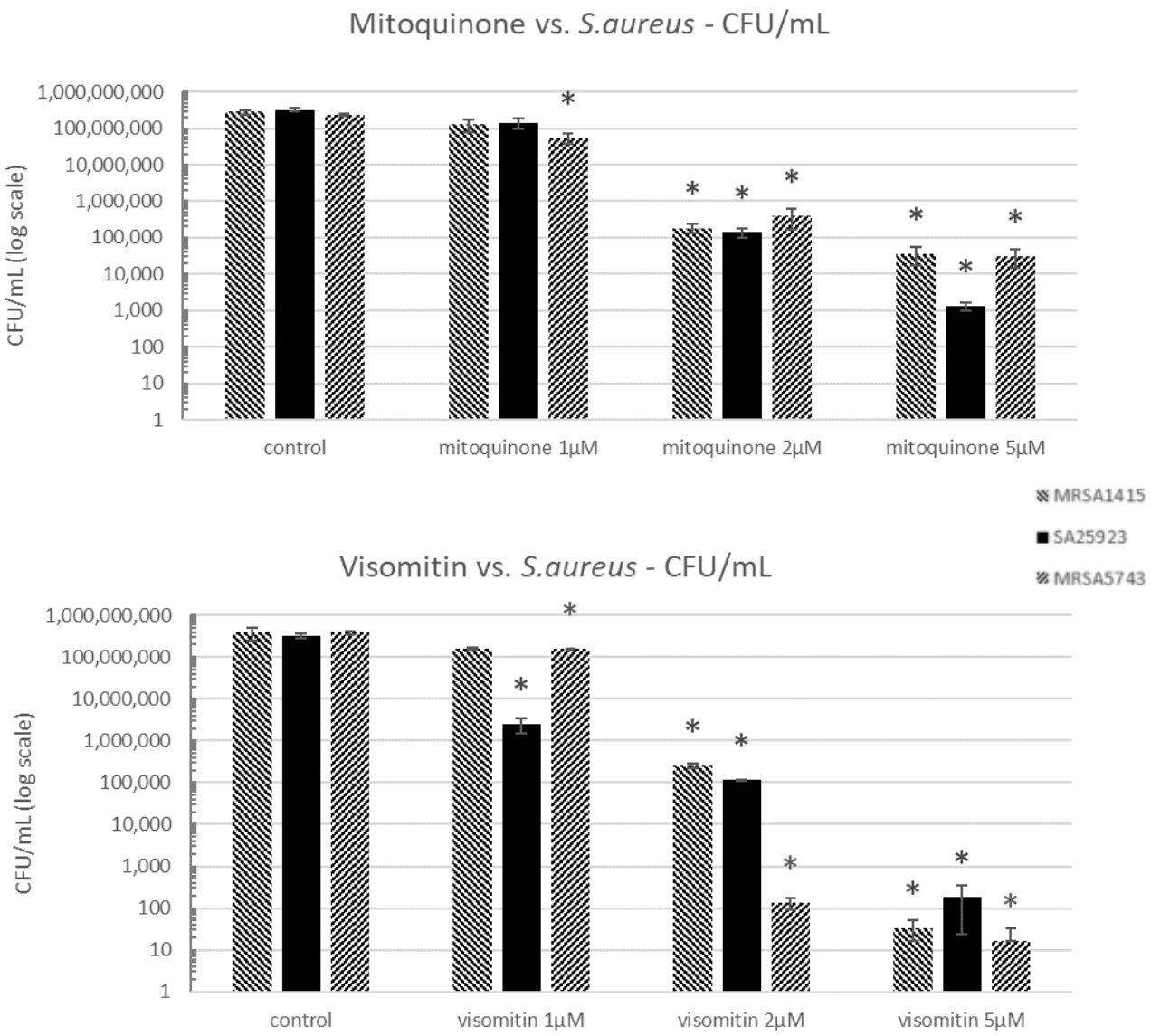


Figure 1: Colony forming units (CFU)/milliliter (y-axis, log scale) after 16 hrs exposure to MTA (mitoquinone and visomitin) at 1, 2 and 5 µM. *S. aureus* ATCC 25923 and 2 MRSA clinical isolates (MRSA1415 and MRSA5748). Error bars represent +/- SEM (3 replicates per strain).
* $p < 0.05$, t-test vs. maximum growth control in broth only, for same bacterial strain.

When these assays were repeated using optical density measurements, the minimum concentrations of mitoquinone needed to suppress visible growth were found to be 2μM for strains SA25923 and MRSA1415, and 5μM for CI MRSA5743. The reductions in growth were also statistically significant compared to maximal growth at 1-5μM for all three strains ($p<0.05$) (Fig. 2).

Visomitin produced a significant reduction of optical density of *S. aureus* 25923 compared to control at 1μM, but not for MRSA1415 and MRSA5743. At ≥ 2μM, visomitin produced a significant reduction in visible growth of all three strains ($p<0.05$) (Fig. 2).

Importantly, there was also no significant difference between positive control and MTA vehicle control (≤10μL 1:1 ethanol:water in 5mL broth).

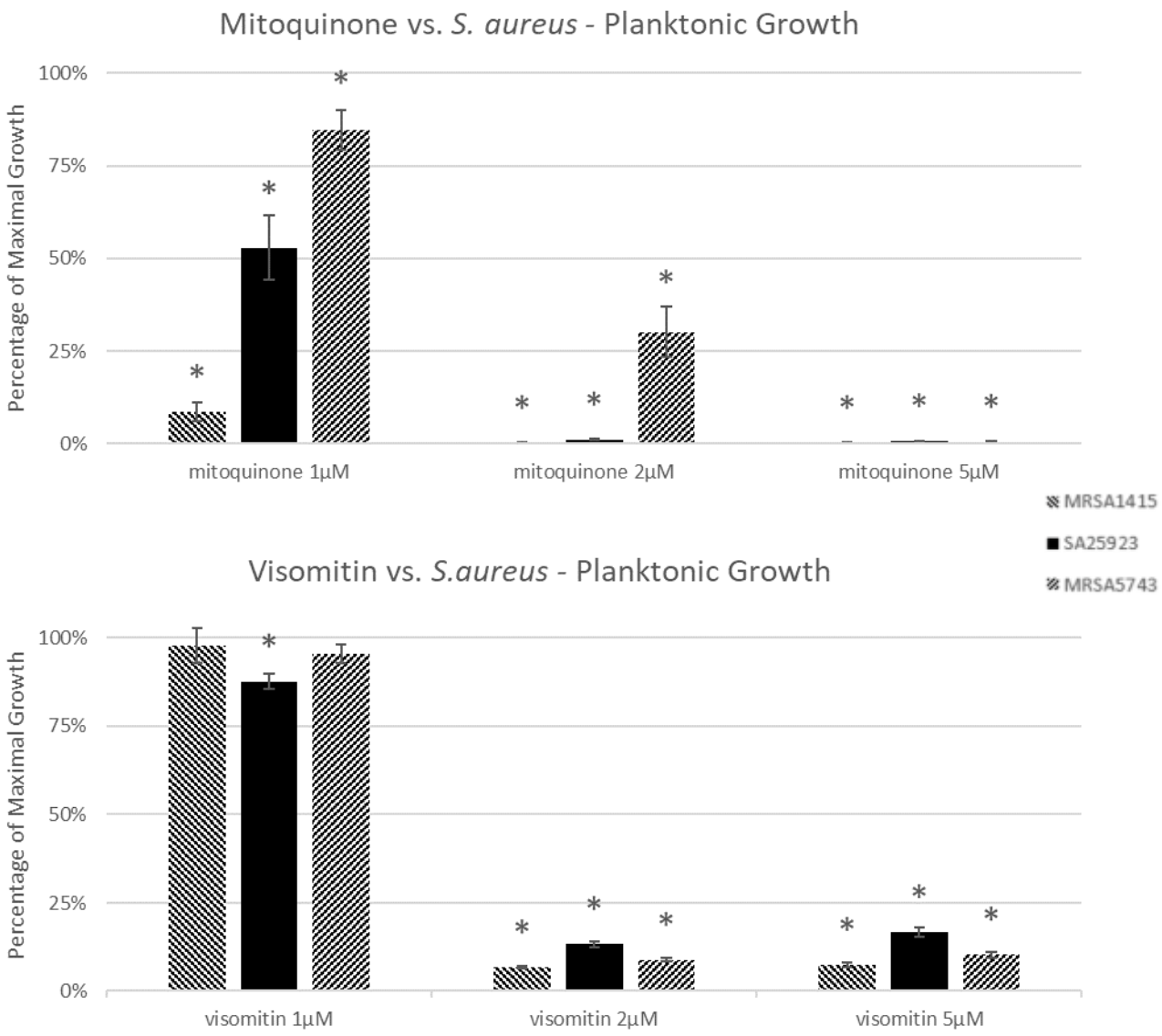


Figure 2: Percentage of maximal planktonic bacterial growth (y-axis) after 16 hours exposure to MTA (mitoquinone and visomitin) at 1µM, 2µM and 5µM.
Error bars represent +/- SEM (3 replicates per strain).
* $p < 0.05$, t-test vs. maximum growth control in broth only, for same strain.

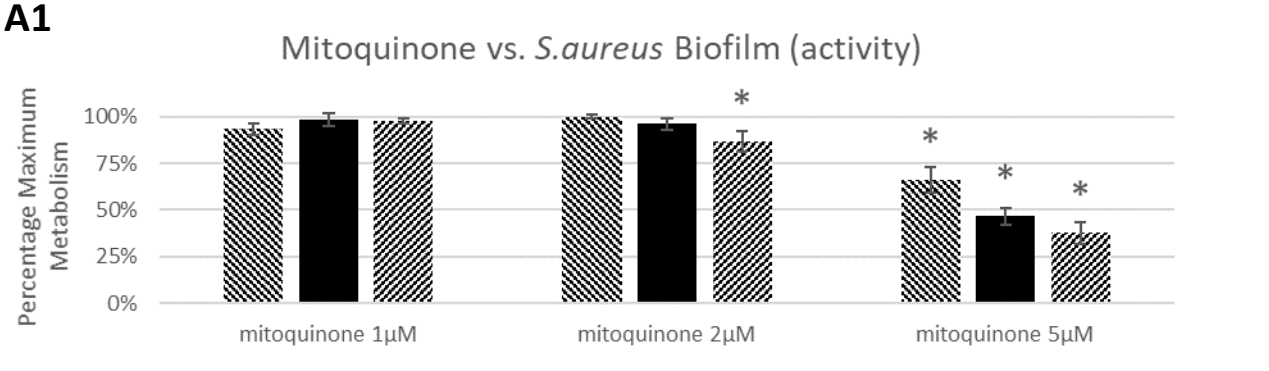
MTAs are active against established *S. aureus* biofilms

Mitoquinone also showed significant activity against 48 hour-old *S. aureus* biofilms after 12 hours of exposure, but only at 5μM ($p<0.05$), which reduced the metabolic activity of the biofilm an average of 51% across the three strains compared to untreated maximal growth control (Figure. 3A1). 5μM mitoquinone treatment also produced a 69% average decrease in biofilm viability across the three strains, as measured with a crystal violet assay ($p<0.05$).

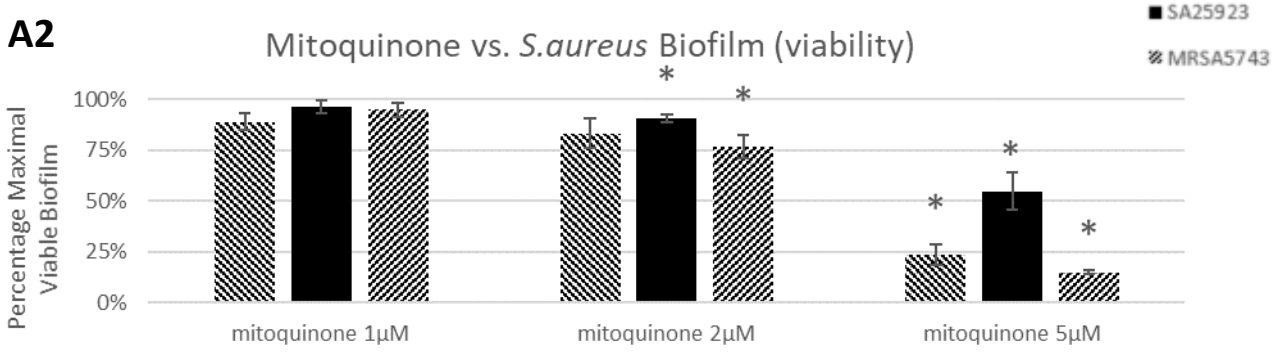
Live/dead imaging after treatment with mitoquinone confirmed a decreased viable biomass as well as physical disruption of the extracellular polymeric substance (Supplementary Figure 1).

Treatment with visomitin also reduced the biofilm metabolic activity at doses ≥ 1μM for strains SA29523 and MRSA1415, and ≥ 2μM for CI MRSA5743. However, unlike mitoquinone, crystal violet assays indicated this change did not appear to be associated with a consistent reduction in *S. aureus* biofilm viability at doses < 5μM (Figure. 3B2):

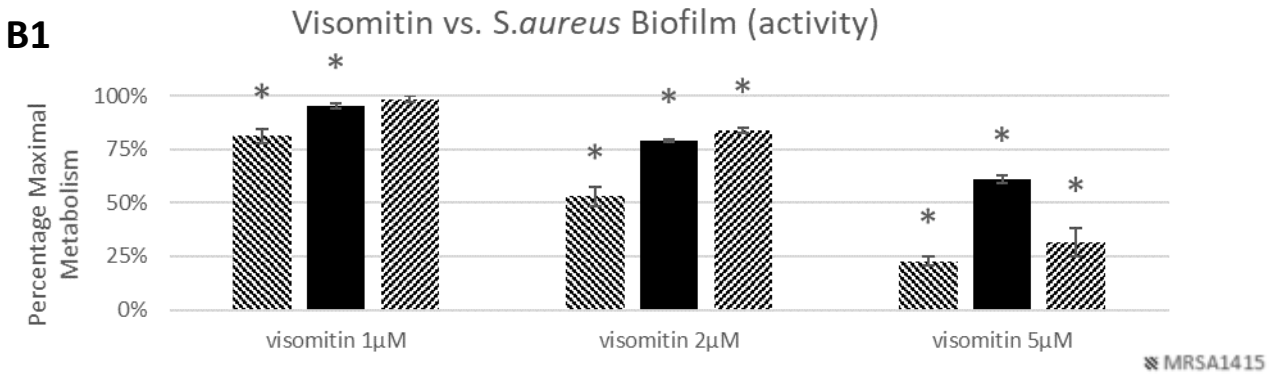
A1


Mitoquinone vs. S.aureus Biofilm (activity)
Percentage Maximum Metabolism
100%
75%
50%
25%
0%
mitoquinone 1µM
mitoquinone 2µM
mitoquinone 5µM
MRSA1415
SA25923
MRSA5743

A2


Mitoquinone vs. S.aureus Biofilm (viability)
Percentage Maximal Viable Biofilm
100%
75%
50%
25%
0%
mitoquinone 1µM
mitoquinone 2µM
mitoquinone 5µM

B1


Visomitin vs. S.aureus Biofilm (activity)
Percentage Maximal Metabolism
100%
75%
50%
25%
0%
visomitin 1µM
visomitin 2µM
visomitin 5µM
MRSA1415
SA25923
MRSA5743

B2

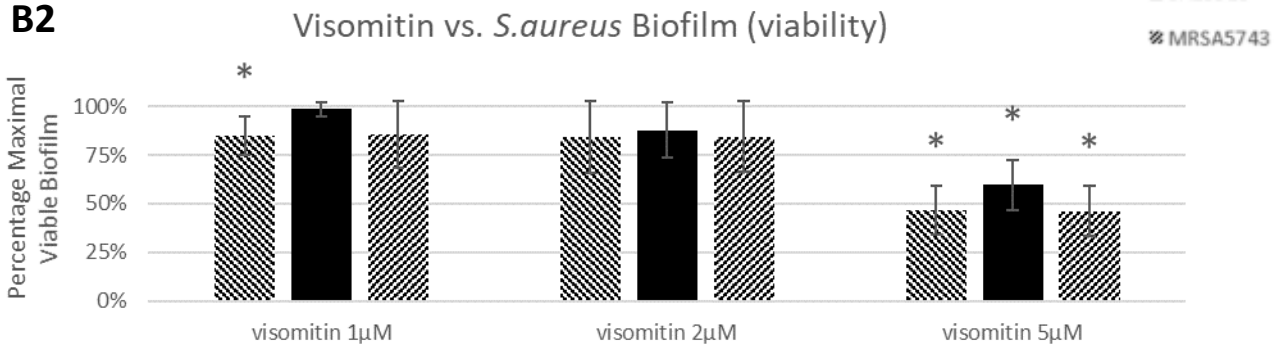
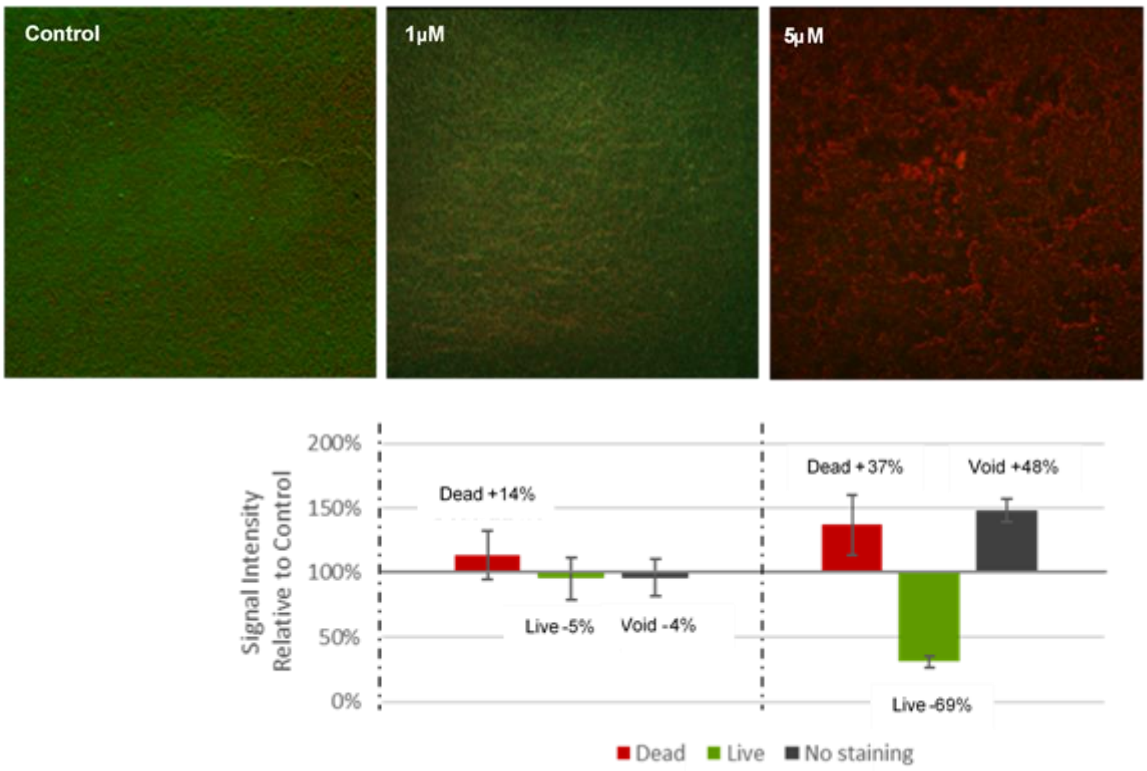

Visomitin vs. S.aureus Biofilm (viability)
Percentage Maximal Viable Biofilm
100%
75%
50%
25%
0%
visomitin 1µM
visomitin 2µM
visomitin 5µM

Figure 3: Alamar Blue assay showing metabolic activity (**A1, B1**) and Crystal Violet assay showing the viability (**A2, B2**) for *S. aureus* and MRSA biofilm treated with MTA (mitoquinone and visomitin) at 1µM, 2µM and 5µM. Results expressed as a percentage of maximal growth control value (broth only) after 12 hours exposure to MTA (mitoquinone **(A)** or visomitin **(B)**).

Error bars represent +/- SEM (3 biological replicates per strain).
* $p < 0.05$, t-test vs. maximum growth control (i.e. broth only), for same bacterial strain.



Supplementary Figure 1: Confocal microscopy images (1280x1280x93µm, 14 transposed slices) of live/dead (green/red) stained, *S. aureus* biofilm after 12 hours exposure to mitoquinone. Error bars represent standard deviation of signal intensities.

The corresponding histogram represents the total increase in dead-stained cells (red), the total decrease in live-stained cells (green) and the increase in unoccupied dead space (black) for each concentration of mitoquinone.

(1uM) resulted in a non-significant increase in dead bacteria and reciprocal decrease in live bacteria (green bar) compared to no-treatment control.
(5µM) killed 37% of bacteria and reduced live bacteria by 69%, with 48% structural disruption and maladhesion (black bar).

Mitoquinone reduces *S. aureus in vivo* without harming the host organism

Mitoquinone reduced CFUs of *S. aureus* ATCC 25923 that had invaded *C. elegans* nematodes from 33898 CFU/organism in the maximal bacterial growth control to 160 CFU/organism in the treatment group (equivalent to a 2 Log reduction). In doing so, it did not alter the survival of the host organisms compared to untreated or infected controls (91% vs. 92% and 89% respectively, $p<0.05$).

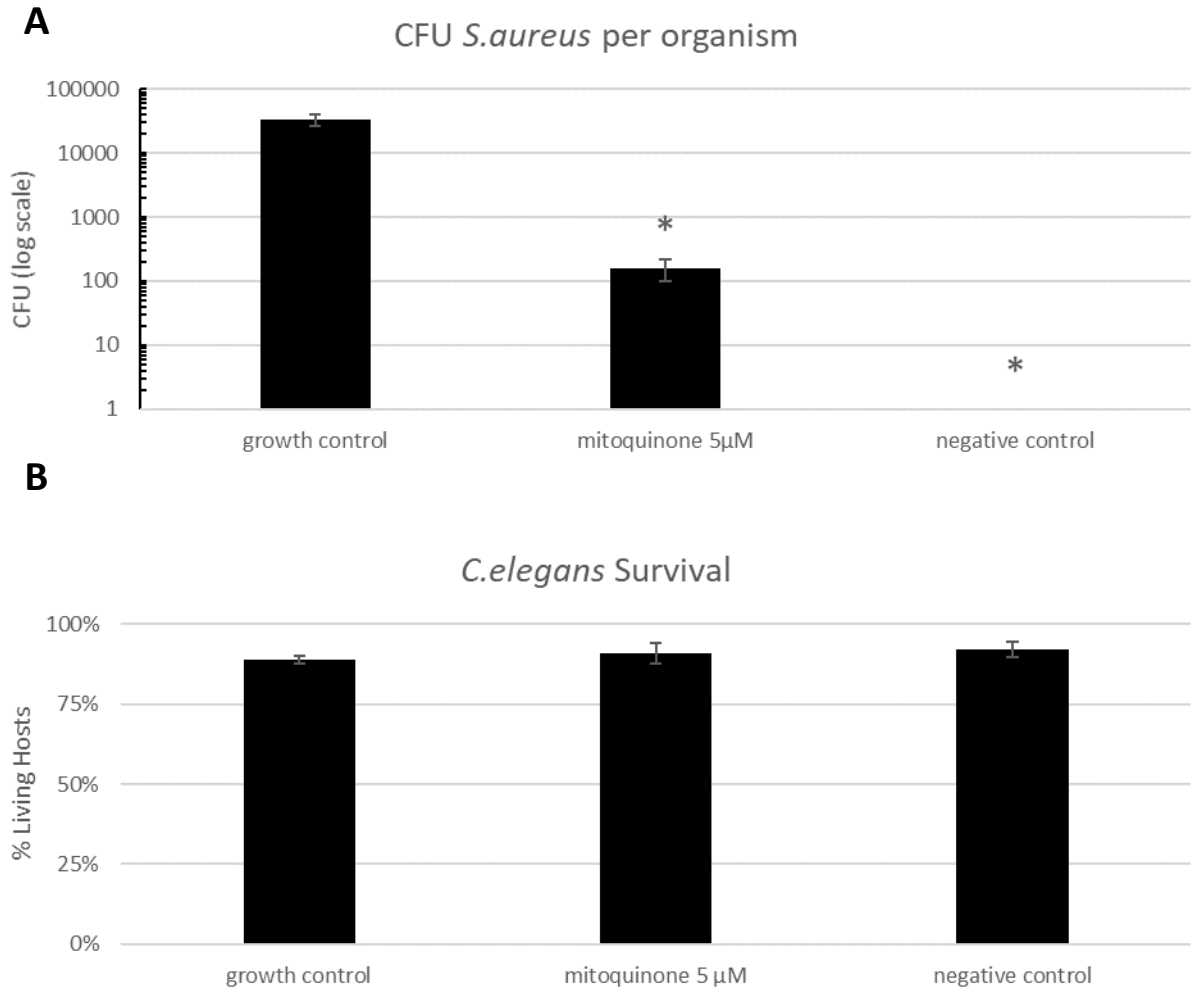


Figure 4: (A) *C.elegans* CFU assay of invasive *S. aureus.*
(B) Host organism survival % for each treatment group.
* $p < 0.05$, t-test vs. maximum growth control (i.e. broth only). Error bars represent +/- SEM.

MTAs synergistically increase the activity of a antimicrobials against planktonic *S. aureus* and its biofilm

Mitoquinone and visomitin both improved the activity of four common antimicrobials from different classes against planktonic *S. aureus.* Both MTA's had already been shown to inhibit visible growth of all three strains of planktonic *S. aureus* at 2μM - 5μM (Figure 2) and this effect was not interrupted after combination with any of the antibiotics:

	Minimum Inhibitory Concentrations (mg/L)											
	***S. aureus* 25923**				**MRSA 5743**				**MRSA 1415**			
Mitoquinone dose	**0**	1μM	2μM	5μM	0	1μM	2μM	5μM	0	1μM	2μM	5μM
MIC of Augmentin (875/125)	0.25	0.13	<0.02	<0.02	>4	2	<0.02	<0.02	>4	<0.02	<0.02	<0.02
MIC of Doxycycline	0.13	<0.02	<0.02	<0.02	0.25	0.13	0.06	<0.02	0.25	<0.02	<0.02	<0.02
MIC of Clarithromycin	0.13	0.03	<0.02	<0.02	0.13	0.03	<0.02	<0.02	0.13	<0.02	<0.02	<0.02
MIC of Mupirocin	0.03	<0.02	<0.02	<0.02	0.02	<0.02	<0.02	<0.02	0.02	<0.02	<0.02	<0.02

	Minimum Inhibitory Concentrations (mg/L)											
	***S. aureus* 25923**				**MRSA 5743**				**MRSA 1415**			
Visomitin dose	**0**	1μM	2μM	5μM	0	1μM	2μM	5μM	0	1μM	2μM	5μM
MIC of Augmentin (875/125)	0.25	0.13	<0.02	<0.02	4	2	<0.02	<0.02	>4	<0.02	<0.02	<0.02
MIC of Doxycycline	0.13	<0.02	<0.02	<0.02	0.25	0.13	<0.02	<0.02	0.25	<0.02	<0.02	<0.02
MIC of Clarithromycin	0.13	0.13	<0.02	<0.02	0.13	0.13	<0.02	<0.02	0.13	<0.02	<0.02	<0.02
MIC of Mupirocin	0.03	<0.02	<0.02	<0.02	0.02	<0.02	<0.02	<0.02	0.02	<0.02	<0.02	<0.02

Table 1: Average minimum inhibitory concentrations for planktonic *S. aureus* as measured after 16 hours combination therapy using serial dilutions of antibiotics combined with three doses of MTA (1μM, 2μM or 5μM) compared with antibiotic alone. Values represent averages of three biological replicates tested for each strain. Grey boxes indicate synergy (ΣFIC < 0.5), white boxes indicate non-synergistic (ΣFIC ≥ 0.5). Red text values denote MTA MIC for that strain. Orange boxes denote ≥ MIC of MTA for that strain.

Taken with the MIC data for mitoquinone and Visomitin (Figure 2), these data were then used to calculate fractional inhibitory concentration (FIC) and ascertain whether combination therapy had a synergistic or additive effect. At the given MTA MIC for each strain, all MTA/antibiotic combinations had an ΣFIC <0.5, indicating a synergistic effect i.e. greater than the sum of its parts. Adding a sub-MIC dose (1-2μM) of either MTA to augmentin, doxycycline and clarithromycin also had a synergistic antimicrobial effect on at least 2/3 strains. Mupirocin did not benefit synergistically from either MTA at any dose, due mostly to its efficacy as a sole agent. Both MTAs did not antagonise any of the antimicrobials' antibiofilm action (i.e. no ΣFIC ≥ 4)

As with planktonic bacteria, MTAs also augmented the activity of various antimicrobials against *S. aureus* biofilm (Figure 4). Combination therapy with mitoquinone doses ≥5μM significantly improved the efficacy of augmentin (amoxicillin and clavulanate) and doxycycline against *S. aureus* biofilms ($p < 0.05$) in an additive (rather than synergistic) fashion, as measured using an Alamar Blue assay. Clarithromycin derived a synergistic efficacy from 2μM of visomitin, but this effect was additive when using 5μM of visomitin. In the case of mupirocin, which had efficacy against biofilm even as a singular treatment (>60% less growth than control at 4mg/mL), only 5μM mitoquinone showed additive benefit, and only at the highest mupirocin concentration tested (4mg/mL):

Combination therapy with visomitin doses ≥2μM significantly improved the efficacy of augmentin, doxycycline and clarithromycin against *S. aureus* biofilms ($p < 0.05$) in an additive fashion. Doxycycline also derived a synergistic efficacy from 2μM of visomitin between 2 and 0.25mg/mL concentrations that was not seen using 5μM of visomitin. As with mitoquinone, mupirocin did not add a discernable additive benefit to visomitin due to its own antimicrobial effectiveness.

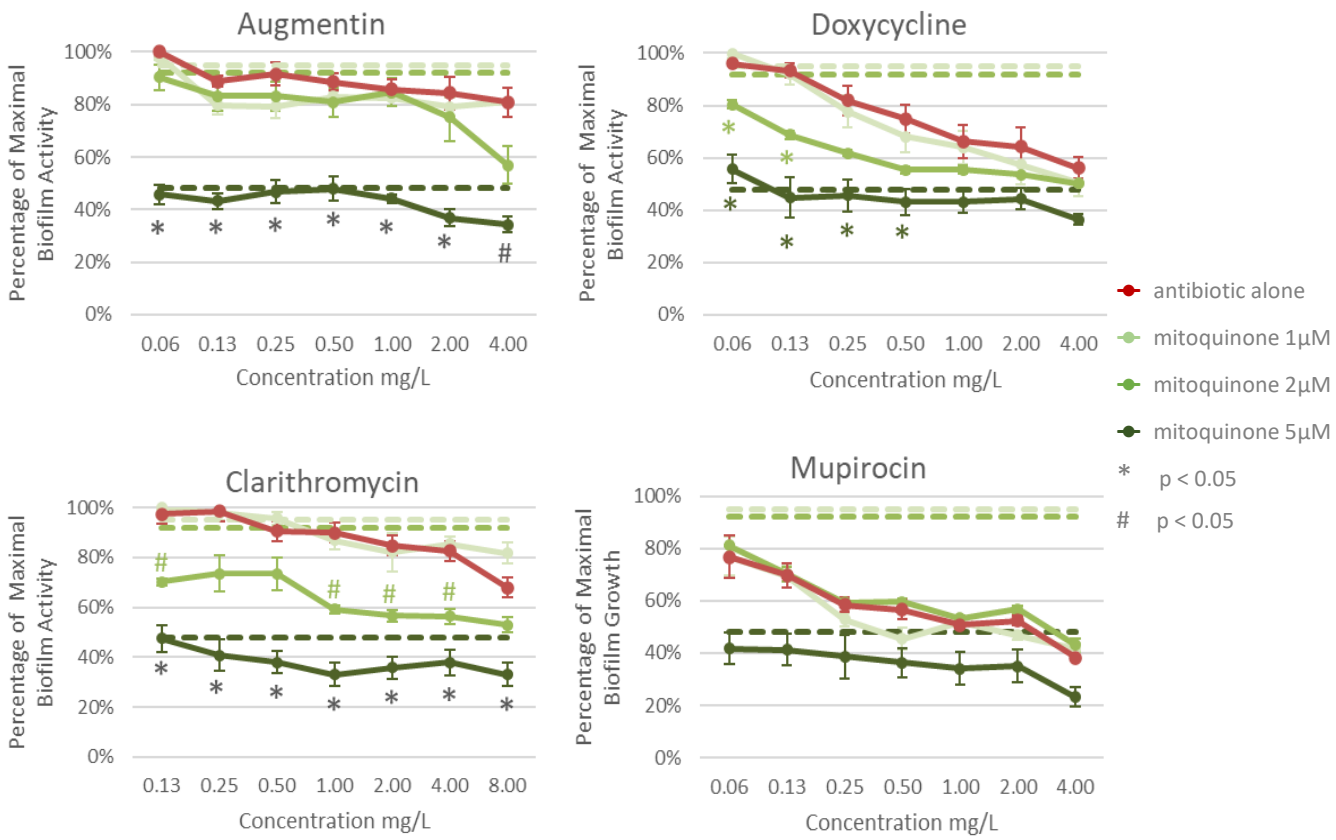


Figure 5a: Percentage of maximal metabolic activity of *S. aureus* biofilm (y-axis) compared to maximal growth control (broth only) for combination therapy using serial dilutions of antibiotics (x-axis) combined with three doses of MTA (colored lines) and with antibiotic alone (red line).
Average values for *S. aureus* ATCC 25923 and 2 MRSA clinical isolates.
Bars represent +/- SEM (3 biological replicates per strain).
Dotted bars represent percentage maximal activity when bacteria were treated with MTA alone.
* significant reduction, $p<0.05$, t-test, compared to antibiotic alone
\# significant reduction, $p<0.05$, t-tests, compared to both antibiotic and equivalent dose of MTA alone.

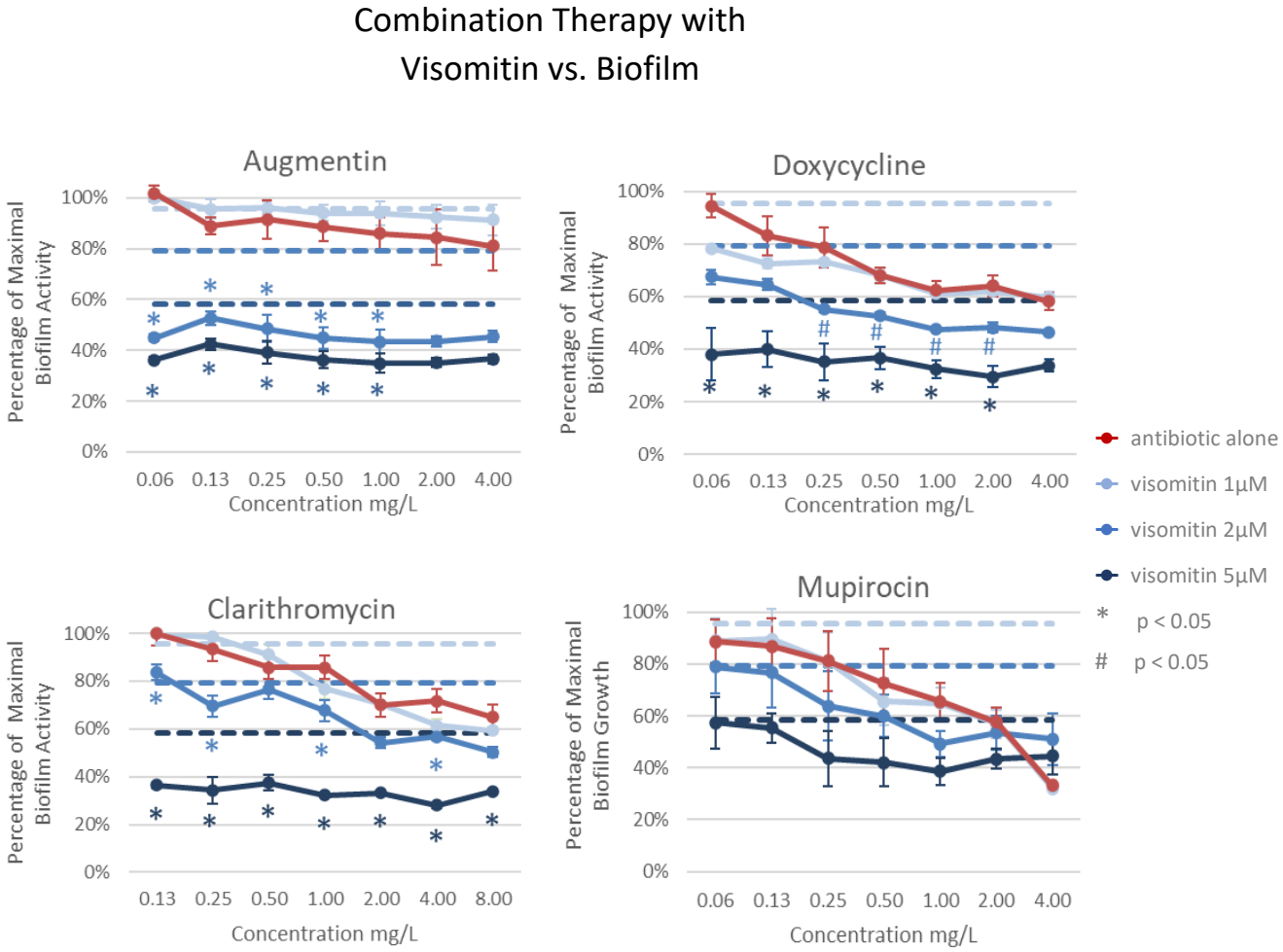


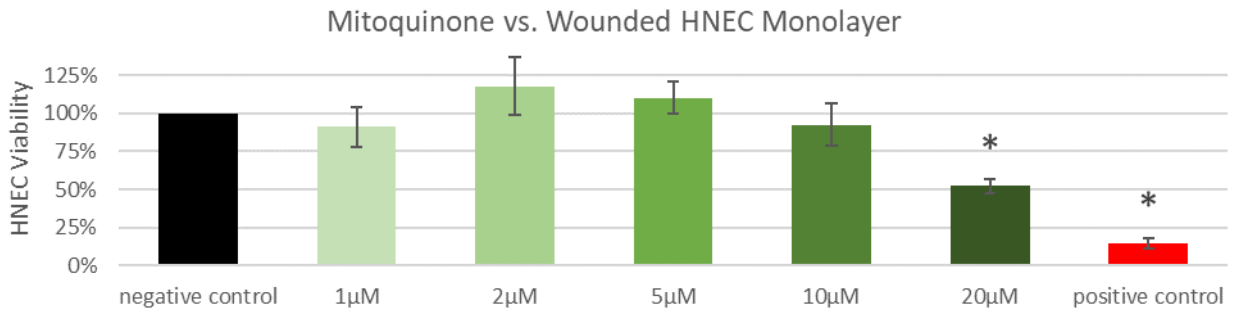
Figure 5b: Percentage of maximal metabolic activity of *S. aureus* biofilm (y-axis) compared to maximal growth control (broth only) for combination therapy using serial dilutions of antibiotics (x-axis) combined with three doses of MTA (colored lines) and with antibiotic alone (red line).
Average values for *S. aureus* ATCC 25923 and 2 MRSA clinical isolates.
Bars represent +/- SEM (3 biological replicates per strain).
Dotted bars represent percentage maximal activity when bacteria were treated with MTA alone.
* significant reduction, p<0.05, t-test, compared to antibiotic alone
\# significant reduction, p<0.05, t-tests, compared to both antibiotic and equivalent dose of MTA alone.

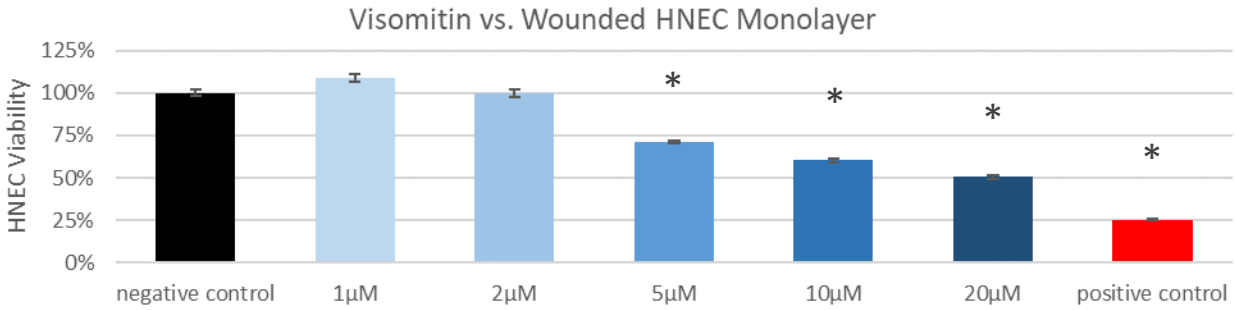
Safety Studies

Low dose MTAs did not induce cytotoxicity

Mitoquinone was not harmful to primary HNEC at doses ≤10 μM or primary human sinonasal fibroblasts at doses ≤5 μM following 48 hours of treatment exposure, as determined by an LDH cytotoxicity assay.

Visomitin showed toxicity against HNEC at a lower concentration than that of mitoquinone (≥5 μM). It had similar toxicity against primary human sinonasal fibroblasts, showing no evidence of cytotoxicity at doses ≤2 μM following 48 hours of treatment exposure:


Mitoquinone vs. Wounded HNEC Monolayer
HNEC Viability
125%
100%
75%
50%
25%
0%
negative control
1µM
2µM
5µM
10µM
20µM
positive control
*
*


Visomitin vs. Wounded HNEC Monolayer
HNEC Viability
125%
100%
75%
50%
25%
0%
negative control
1µM
2µM
5µM
10µM
20µM
positive control
*
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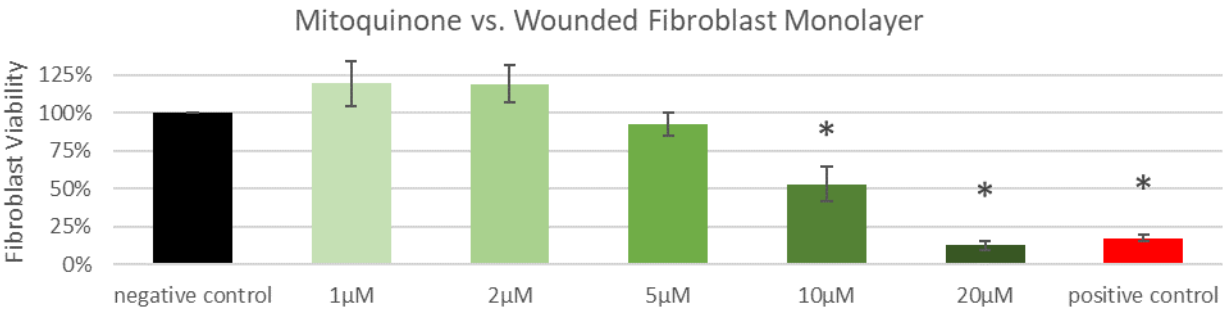
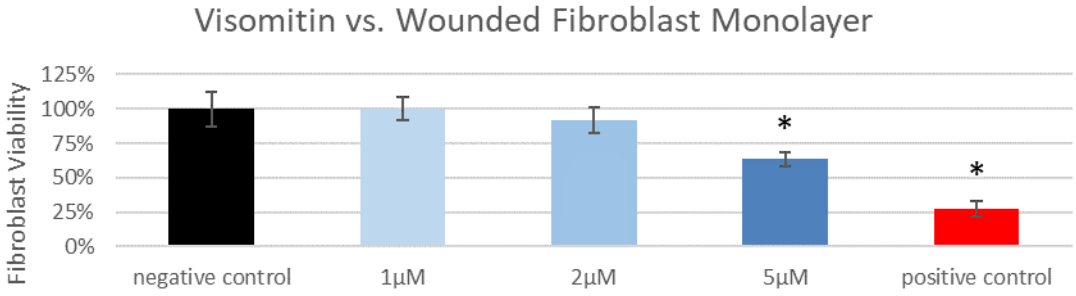

Mitoquinone vs. Wounded Fibroblast Monolayer
Fibroblast Viability
125%
100%
75%
50%
25%
0%
negative control
1µM
2µM
5µM
10µM
20µM
positive control
*
*
*


Visomitin vs. Wounded Fibroblast Monolayer
Fibroblast Viability
125%
100%
75%
50%
25%
0%
negative control
1µM
2µM
5µM
positive control
*
*

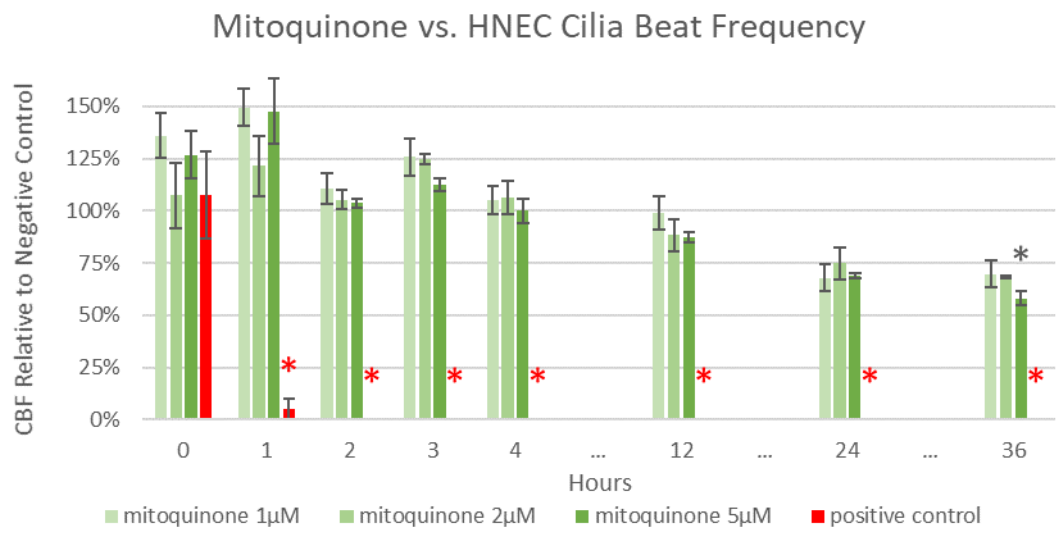
Supplementary Figure 2: primary HNEC and human fibroblasts and LDH release after 48 hours exposure to mitoquinone, compared with cell media alone (negative control). Cell viability (y-axis) calculated as % LDH release compared with negative control.
* $p < 0.05$, Students t-test vs. negative control.
Bars represent +\- SEM (3 patients with 2 technical replicates).

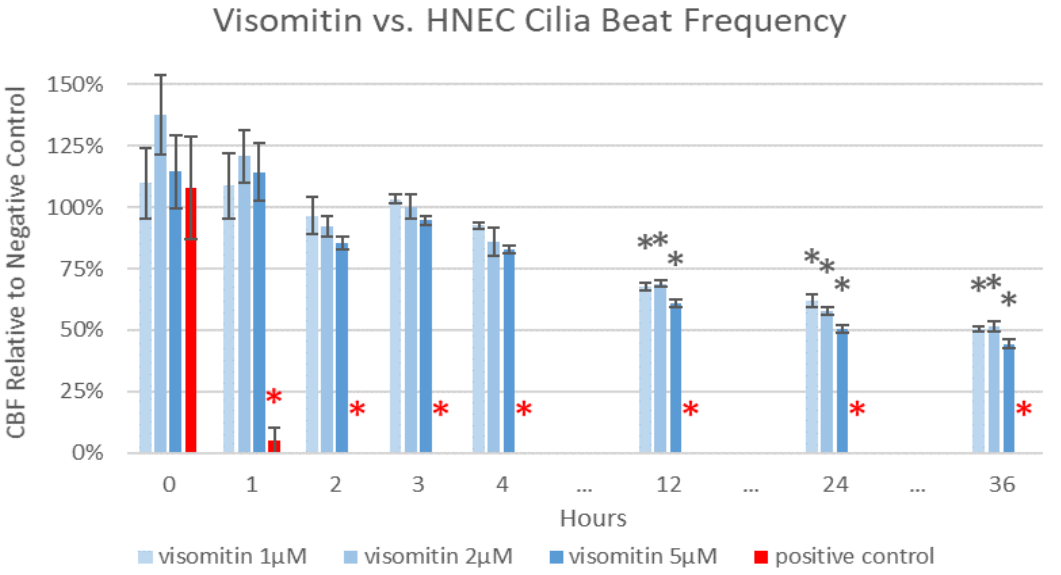
Low dose mitoquinone did not alter HNEC cilia beat frequency, although visomitin did so from 12 hours

Compared to media alone (negative control), mitoquinone did not consistently alter cilia beat frequency (CBF) in mature (> 8 weeks at air liquid interface culture), polarized, primary HNEC over 24 hours of topical submersion ≤ 5µM (Supplementary Figure 3). After 24 hours, mean normalized CBF for 1µM, 2µM and 5µM were 68%, 75% and 69% of negative control, respectively. At 36 hours, only 5µM mitoquinone had significantly slowed CBF compared to negative control.

Visomitin significantly slowed CBF from at least 12 hours at all doses (1µM, 2µM and 5µM). After 24 hours, mean normalized CBF for 1µM, 2µM and 5µM were 62%, 58% and 51% of negative control, respectively.

All cilia variably increased activity in response to their initial submersion with 400µL PBS (0-1hrs), however, this variability resolved from 2 hours. Cilia did not tolerate >36 hours vehicle submersion over their apical (normally air-facing) surface, limiting the reliability of this model at that time point:





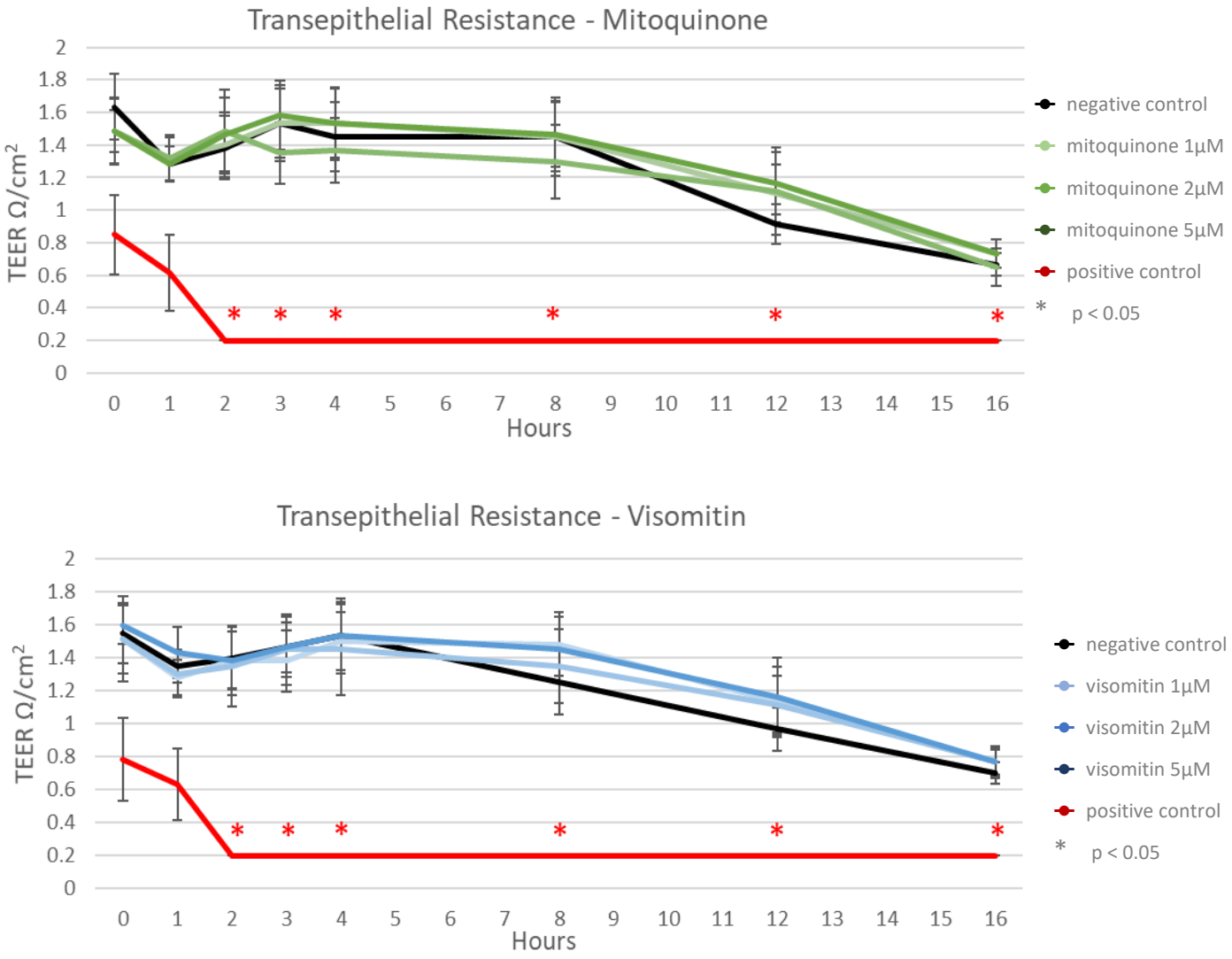
Supplementary Figure 3: primary human nasal epithelial cells cilia beat frequency after 36 hours exposure to topical MTA, compared with cell media alone (negative control).
3 cell lines, 2 technical replicates each. * $p < 0.05$, t-test vs. negative control. Bars represent +\- SEM (3 patients with 3 technical replicates).

Both MTAs did not alter HNEC trans-epithelial electrical resistance or membrane permeability at non-toxic doses

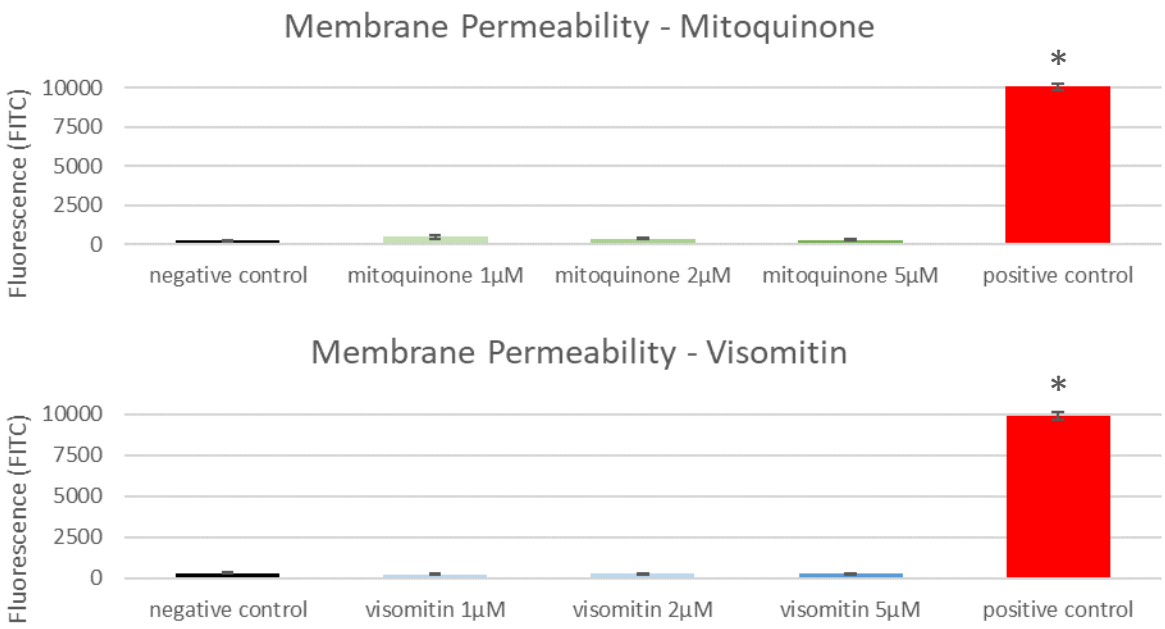
Compared to vehicle control (PBS alone), both mitoquinone and visomitin did not alter trans-epithelial resistance in mature (> 8 weeks at air liquid interface culture), polarized, primary HNEC over 16 hours of topical submersion ≤ 5μM.

Use of a FITC-dextran permeability assay confirmed membrane integrity after 16 hours exposure to either treatment at 1μM, 2μM and 5μM.

Membrane integrity for both treated and untreated cells began to decrease rapidly after 16 hours due to PBS vehicle submersion over their apical (normally air-facing) surface, limiting the reliability of this model after that time point:



Supplementary Figure 4: Primary human nasal epithelial cells trans-epithelial electrical resistance after 16 hours exposure to topical MTA, compared with cell media alone (negative control).
3 cell lines, 2 technical replicates each. * $p < 0.05$, t-test vs. negative control.
Bars represent +\- SEM (3 patients with 3 technical replicates).



Supplementary Figure 5: Fluorescence of media in basal chamber 2 hours after loading fluorescent FITC-dextran into apical chamber. This data represents primary human nasal epithelial cells membrane permeability after 16 hours exposure to each MTA, compared with cell media alone (negative control).
3 cell lines, 2 technical replicates each. * $p < 0.05$, t-test vs. negative control.
Bars represent +\- SEM (3 patients with 3 technical replicates).

DISCUSSION

Mitoquinone, much like its fellow MTA visomitin, demonstrated good efficacy against staphylococcal biofilms and their planktonic counterparts. When tested against primary human nasal epithelial and fibroblast cell cultures from several patients they did not demonstrate toxicity or induce dysfunction at their effective antimicrobial doses. They also demonstrated additive anti-biofilm efficacy with existing antimicrobials, which was occasionally synergistic at lower doses.

Both MTAs have previously been shown to have antimicrobial activity against a range of planktonic bacteria. [255] The fact that the MIC of these agents against various gram-negative and gram-positive bacteria consists of single digit, micromolar concentrations points to a high permeability of these agents through the bacterial cell envelope. [255] The antibacterial action of MTAs has previously been attributed to the activity of their alkyl trimethyl, alkyl trihexyl and alkyl triphenyl phosphonium salts [263, 264] which have the ability to disturb the bacterial membrane in a similar fashion to that of quaternary ammonium compounds, such as cetrimide. [265, 267] This action leads to a generalized and progressive leakage of cytoplasmic material, causing bacteria to lose osmoregulatory capability. MTA bactericidal action may also involve suppression of bacterial bioenergetics, by collapsing membrane potential via protonophorous-like uncoupling. [255, 268-270, 320] The fact that this action efficaciously suppresses growth of a significant and highly persistent pathogen like *S. aureus* signals MTAs' novel therapeutic potential in the post-operative setting, where recolonisation may lead to adverse outcomes and should be aggressively counteracted.

Of some concern is the fact that combining antioxidants and antibiotics theoretically has the potential to reduce the antimicrobial efficacy of the antibiotic - particularly bactericidal antibiotics, given that their antibiotic–induced ROS formation is thought to facilitate bacterial killing. [211, 231] Indeed, non-specific antioxidants, such as the ROS-quenching antioxidant thiourea, have been shown to reduce the bacterial killing efficacy

of bactericidal antibiotics. [231, 232] In direct contrast, there is emerging evidence that mitoquinone may augment the activity of existing antibiotics [229] due to the membrane disruption caused by its cation moiety. Whilst the present study adds evidence for its efficacy against gram-positive bacteria, some studies have shown that MTAs are less effective against gram-negative and anaerobic bacteria. [229, 255] One explanation may be the presence of certain transporters, such as AcrAB-TolC in some strains such as *E. coli*, that actively facilitate efflux of MTAs. [255] However, unlike other antioxidants, the activity of antimicrobials against gram-negative pathogens like *Pseudomonas aeruginosa* and *Haemophilus influenzae* does not appear to be compromised by MTAs at pharmacological levels [229]. This means that the issue of MTA resistance could potentially be circumvented by combination therapy of MTAs with appropriate antimicrobials that target gram-negative and/or anaerobic bacteria.

Given the potent antibacterial activity observed in the present study, it was important to investigate any collateral cytotoxicity of MTAs towards sinonasal cells. The lack of cytotoxicity seen after 48 hours exposure at concentrations ≤ 5μM was supported by the healthy cell morphology and activity observed during confocal microscopy. At ≤ 5μM, mitoquinone was also found to be non-toxic to primary human nasal epithelial cells grown at air-liquid interface; providing a high-fidelity model of its *in vivo* safety in terms of cell viability, cilia beat frequency and cell barrier integrity. Doses ≥ 20μM and ≥ 10μM were found to be toxic to HNEC and fibroblasts respectively, potentially indicating that the level of ROS suppression caused by these doses was critically detrimental to the normal housekeeping function of ROS within the cell. [191, 251]

Mitoquinone has previously shown a robust safety profile with human tissue both *in vitro* [255] and during multiple phase II human trials. [246, 272, 274] In another study, visomitin also did not display any significant toxicity against HeLa cells exposed to ≤ 5μM concentrations for 17 hours. [255] Composite membranes loaded with up to 80μM of mitoquinone have also been used in animal models without adverse effects, although the

precise release profile of the mitoquinone was somewhat unclear. [321] As such there is good preliminary evidence to support safe use of MTAs *in vivo* at doses used in the present experiment.

Whilst these findings are currently translational in nature, the present study is a necessary step in the move towards *in vivo* experimentation utilizing models with a functioning immune system. Given the positive safety and efficacy profile *in vitro,* MTAs such as mitoquinone and visomitin warrant further therapeutic investigation in settings that are prone to *S. aureus* biofilm formation.

CONCLUSION

Successful treatment of bacterial biofilms *in vivo* hinges on both environmental and host factors. The mitochondrially-targeted antioxidants mitoquinone and visomitin contain both an effective antioxidant core targeting mitochondrial reactive oxygen species and a strong antimicrobial moiety. This combination makes it a potent treatment for biofilms in the upper respiratory tract, where infection and inflammation are often comingled. The absence of cytotoxicity for human cells and its effectiveness even at micromolar concentrations means that MTAs hold great promise for medical treatment of biofilms alone, or in combination with existing antimicrobials.

Statement of Authorship

Title of Paper	*In Vitro* Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Reducing Adhesions after Sinus Surgery
Publication Status	Prepared for Submission
Publication Details	Withheld Under Embargo

Principal Author

Name of Principal Author (Candidate)	Dr Michael Gouzos		
Contribution to the Paper	Project design, experimental work, data collection and analysis, manuscript preparation		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/12/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Project design, manuscript preparation		
Signature		Date	7/12/20

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Contribution to the Paper	Project design, manuscript preparation		
Signature		Date	7/12/20

Name of Co-Author	A/Prof. Sarah Vreugde		
Contribution to the Paper	Project design, manuscript preparation		
Signature		Date	7/12/20

In Vitro Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Reducing Adhesions after Sinus Surgery

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Short title: Mitoquinone Improves Wound Healing

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Supplementary Figures: 4

Abstract

Background Favorable postoperative outcomes after sinus surgery hinge on optimizing the quality of mucosal healing. This is evidenced by the fact that adhesions and scar formation are frequently observed in patients eventually requiring revision surgery. The present study aims to evaluate the *in vitro* safety and wound healing efficacy of treating wounded sinonasal cells with mitochondrially-targeted antioxidants (MTA) to reduce the risk of adhesion formation.

Methods A dose-response curve of cell migration and mitochondrial Reactive Oxygen Species (ROS) production in nasal fibroblasts was established for 1-20μM of mitoquinone using time-lapse confocal laser scanning microscopy (CLSM). These experiments were also repeated using human nasal epithelial cells (HNECs) and with a second MTA, visomitin. Key concentrations were also tested for cytotoxicity, cilia function and barrier integrity.

Results Mitoquinone showed a significant ($p<0.05$) dose-dependent slowing of fibroblast migration at all doses (1-20μM), and a significant reduction in ROS production at higher doses (10-20μM). This effect was mirrored to a lesser degree by visomitin. Neither MTA had a detrimental effect on HNEC migration. Both agents did not damage cellular function at doses <5μM.

Conclusions The effective, non-toxic dose range of MTAs mitoquinone and visomitin was approximately 2-5μM. This absence of cytotoxicity and effectiveness even at micromolar concentrations means that MTAs hold great promise for improving wound healing outcomes in the postoperative setting.

INTRODUCTION

Development of post-operative nasal adhesions is the most common complication of nasal and sinus surgery. [121, 122] Rates are typically reported after 3-5 years and range between 15-30% in randomised controlled trials and between 3-36% in retrospective reviews. [122, 123] Adhesions are known to interfere with the normal mucociliary transport, resulting in pooled mucous which is an ideal growth medium for a variety of microbial pathogens. [121] Where they narrow or obstruct ostia, adhesions and synechiae may cause pain from pressure of retained secretions in addition to predisposing to recurrent disease. [93, 121] In those patients requiring revision surgery, various authors describe adhesions as being a causative factor of surgical failure in up to 60% of these cases. [123-125] Although the prevalence of scars, adhesions and synechiae are multifactorial and may differ greatly between surgeons and centers, there is significant interest in developing perioperative adjuncts that can minimize their incidence and severity.

The role of reactive oxygen species (ROS) in the pathogenesis of cell death and tissue damage on undamaged cell monolayers also has significant implications for its role in wound healing. [194-196] Various models of wound healing have demonstrated that the most potent ROS in wound-healing and postoperative inflammation is hydrogen peroxide (H_2O_2). [197] *In vitro* studies have demonstrated that micromolar concentrations of ROS stimulate the proliferation of human fibroblasts and vascular endothelial cells, whilst also acting as a chemoattractant to inflammatory cells, independent of any blood-bound signalling components. [200-202] This effect has also been seen *in vivo*, as scavenging ROS after abdominal surgery significantly inhibited postoperative adhesion formation. [203]

Endogenous cellular ROS arise from either the ETC in the inner membrane of the mitochondria, or from a class of enzymes known as oxidoreductases. ROS acquire electrons from other nearby molecules via a redox reaction, which damages the structure of the latter. There is evidence to suggest that basal ROS levels are crucial for normal

cell functioning and homeostasis and aberrantly low levels of ROS induce cell cycle arrest. [170, 171] As mitochondrial oxidative damage contributes to a wide range of human diseases, recent efforts have been made to develop antioxidants designed to accumulate within mitochondria *in vivo*. The most prominent of these mitochondrially targeted antioxidants (MTAs) is mitoquinone, an antioxidant ubiquinone moiety (co-enzyme Q_{10}) covalently attached to a triphenylphosphonium cation. [247] A similar MTA named visomitin (SkQ1) has also shown promise in the treatment of inflammation associated with ocular surface diseases such as dry eye disease and corneal wounds, and is already being used therapeutically for this purpose. [313]

Despite the interest in these agents and their highly pertinent mechanism of action, they have, to date, not been investigated as wound healing adjuncts in sinonasal mucosa. The present study aimed to explore the *in vitro* safety and efficacy of using MTAs mitoquinone and visomitin to limit migration of fibroblasts into a standardized wound. A dose-response relationship and toxicity threshold was also sought as a secondary outcome. Wound healing experiments were also repeated on human nasal epithelial cells (HNECs) to ensure the wounds would still epithelialize, rather than remain open and potentially ulcerate.

MATERIALS & METHODS

Study population

This study was performed in accordance with guidelines approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital and the University of Adelaide (reference HREC/15/TQEH/132). All patients that donated sinonasal cells and/or bacteria gave written, informed consent and all samples obtained were anonymised and coded before use. All patients recruited to the study were undergoing endoscopic sinus surgery for chronic rhinosinusitis (CRS), diagnosed using well established criteria [3, 4]. Exclusion criteria included active smoking, age less than 18 years, pregnancy, systemic immunosuppressive disease and underlying malignancy.

Preparation of Treatments

Mitoquinone mesylate (MedKoo Biosciences Inc., South Carolina, USA) was prepared as high concentration stock (3mg/mL) in 1:1 ethanol: milliQ water as per the manufacturer's instructions. All wound treatments (1µM - 20µM) were formulated using <1% dilutions of this stock in cell media, and all antimicrobial treatments (1µM - 5µM) were formulated using <0.2% dilutions in bacterial broth. These dilutions were also assessed as a vehicle control.

Key experiments were repeated using Visomitin bromide (MedKoo Biosciences Inc., South Carolina, USA). This was prepared as high concentration stock (3mg/mL) in 1:1 ethanol: milliQ water as per the manufacturer's instructions, in an identical fashion to mitoquinone.

Wound Healing

Harvesting and culturing primary human nasal fibroblasts *in vitro*

Sinonasal tissue was biopsied from paranasal sinus mucosa and transferred to a culture plate with Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, UK) supplemented with L-glutamine, fetal bovine serum (FBS, Sigma, St Louis, USA) and penicillin streptomycin (Gibco, Life Technologies, New York, USA). Every 2-3 days, the tissue was washed gently with phosphate-buffered saline (PBS) and medium was replaced with fresh medium until fibroblasts became confluent after approximately 2 weeks. Confluent fibroblasts were washed with PBS, trypsinized and collected using centrifugation. Centrifuged pellets were resuspended in PBS along with Dynabeads Epithelial Enrich (Invitrogen, New York, USA). The tube was wrapped in parafilm and placed on a rotor mixer for 20 minutes at room temperature. Supernatant containing fibroblasts was then transferred to a tissue culture flask (Nunc, Roskilde, Denmark).

Harvesting and air-liquid interface culturing of human nasal epithelial cells *in vitro*

Primary human nasal epithelial cells (HNECs) were harvested from nasal mucosa using an established protocol. [285] Cells were suspended in PneumaCult™-Ex Plus Medium (STEMCELL Technologies Australia, Tullamarine, Australia) and depleted of macrophages using anti-CD68 (Dako, Glostrup, Denmark) before being cultured with Ex Plus Medium in collagen coated flasks at 37°C with 5% CO_2. HNECs were grown until confluent and seeded onto collagen coated 6.5 mm permeable Transwell plates (Corning Incorporated, Corning, USA) at a density of 5×10^4 cells per well. Cells were maintained with Ex Plus medium for 2 days in a cell incubator at 37°C with 5% CO_2. On day 3 after seeding, the apical media was removed and the basal media replaced with PneumaCult™-ALI differentiation media (STEMCELL Technologies Australia, Tullamarine, Australia), exposing the apical cell surface to the atmosphere. HNEC at air liquid interface (HNEC-

ALI) were maintained for a minimum of 21 days prior to experimentation for development of tight junctions and ciliation. [286]

Wound healing (cell migration) assay

In the fibroblast wound closure assay, fibroblasts were seeded between passages 5 and 8 into 24 well plates and allowed to reach 80% confluence over 24 hours. A straight vertical scratch was made down through the fibroblasts and HNEC-ALI cell monolayers by using a 200 µl pipette tip in a reproducible fashion. The media and cell debris was aspirated carefully and culture media containing different concentrations of MTA or media only (negative control) added to each well for 40 hours. The wound closure (cell migration) was recorded using time-lapse LSM700 confocal scanning laser microscopy (Zeiss Microscopy, Germany) at 37ºC in an enclosed chamber with 5% CO_2. Images were recorded every 30 minutes for 4 hours, and then every 4 hours for a further 36 hours. Wound area in pixels was quantified manually for each image using ImageJ Software (v1.52a, National Institutes of Health, USA).

Evaluation of cellular reactive oxygen species activity

Reactive oxygen species (ROS) were detected using a chemiluminescent probe: carboxylated 2′, 7′dichlorodihydrofluorescein diacetate (H2-DCFDA; Invitrogen Life Technologies, Carlsbad, CA, USA) that has been widely validated in the literature on oxidative stress [32]. This carboxylated analogue of H2-DCFDA increases intracellular retention of the molecule, making it suited to longer time-lapse studies [32]. Primary nasal fibroblast cells were cultured in phenol-red free DMEM with 10% FBS, seeded into black 24-well plates (Corning Incorporated, Corning, USA) and at a density of 5×10^5 cells per well and incubated for 24 h in a humidified incubator with 5% CO^2 at 37°C. Fibroblasts and HNEC-ALI cultures were washed with PBS and 10 µM of H2-DCFDA was added for 1 h, at 37°C in the dark. Cells were then washed with PBS and exposed to scratching injury as described above. The fluorescence intensity was recorded using time-lapse LSM700

confocal scanning laser microscopy (Zeiss Microscopy, Germany) using filter range Ex/Em: 492/525 nm every 4 hours for 48 hours.

Safety Studies

Cytotoxicity studies

Primary human fibroblasts or HNECs were maintained in a fully humidified incubator with 5% CO_2 at 37°C prior to cytotoxicity studies. Cells were wounded as described above and exposed to graded micromolar concentrations of MTA or control (cell medium for the negative control and 2% Triton ×100 for the positive control) for 48 hours, followed by determination of lactate dehydrogenase (LDH) with a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA). Briefly, 50 µL of the supernatant from each well was mixed with 50 µL of assay reagent and incubated for 30 minutes in the dark at room temperature. After addition of stop solution, the optical density (OD) was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) and compared across treatment and control groups. Medium alone was used as the negative control and an LDH standard included with the detection kit was tested for quality assurance. Cell viability was determined by comparing LDH release of the untreated control with that of treatment groups.

Cilia Beat Frequency

Cilia Beat Frequency (CBF) of HNEC-ALI cultures was assessed using a 20× objective, and 1.5x magnification on an inverted microscope (Olympus IX70, Tokyo, Japan). Video was recorded using a Model Basler acA645-100µm USB3 camera (Basler AG, Ahrensburg, Germany) at 100 frames per second at a resolution of 640 × 480 pixels. The recorded video samples were analyzed using the Sisson-Ammons Video Analysis (SAVA) system. All measurements were taken at room temperature. A baseline CBF was taken prior to addition of the experimental conditions. Initially HNEC-ALI cultures were washed with 100 µL of PBS then 40 µL of PBS was added to the apical compartment. Cells were

treated with incremental doses of MTA (mitoquinone or visomitin) or control (ALI medium for the negative control and 2% Triton ×100 for the positive control). Baseline measurements were taken from 2 separate regions from each culture. The apical PBS was removed and either the control media, vehicle only control, or mitoquinone treatment was added. Measurements were taken every 4 hours for 36 hours. Results are expressed as a percentage change in CBF (Hz) compared to untreated cells exposed to the same vehicle (PBS).

Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) of HNEC-ALI cultures was measured using an EVOM2 epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL, USA) and results were recorded as a differential in ohms between the apical and basal chambers. 100 µL of PBS (1x) medium was added to the apical chamber of ALI cultures to form an electrical circuit across the cell monolayer and into the basal chamber. Cultures were maintained at 37°C during the measurement period using a heating platform. The corresponding MTA treatment or control (ALI medium for the negative control and 2% Triton ×100 for the positive control) was added to the bottom chamber of each well, and TEER measurements were obtained hourly for 4 hours, then 4-hourly until 16 hours.

FITC-Dextran membrane integrity

After completion of the required treatments on HNEC-ALI cultures, FITC-dextran (Sigma Aldrich, Steinheim, Germany) was added to a final concentration of 0.3 mg/ml in 100 µL PneumaCult-ALI medium to the apical chamber of the transwell plate. Cells were then incubated 37 °C, protected from light, for 2 hours. 40 µL of the media from the bottom (basal) chamber was then transferred to a 96 well plate and its fluorescence read on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) using wavelengths 485 nm and 520 nm for excitation and emission respectively.

Statistical analysis

For experiments utilizing measures over multiple timepoints (wound healing, CBF, TEER), a repeated-measures ANOVA and Dunnett's *post hoc* test was used for statistical analysis to compare treatments with controls.

For experiments comparing treatment groups at a final time point (bacterial studies, LDH assay etc.), groups were compared to controls using a two-tailed Student's t-test. Data are presented as the mean ± SEM unless otherwise stated.

These tests were performed using SPSS software (IBM, U.S.A., v26). Statistical significance was defined as a p-value of less than 0.05.

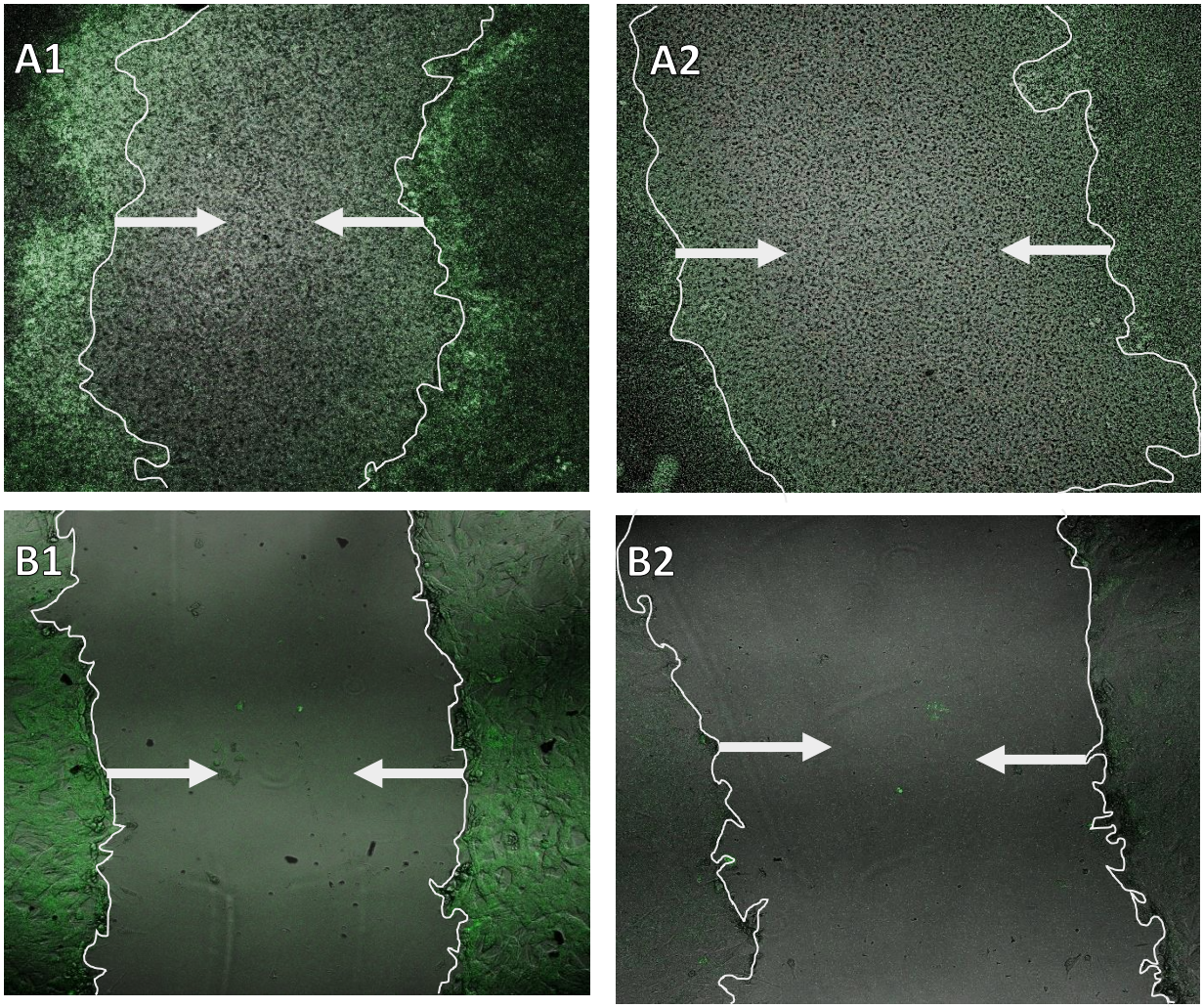
Results

Wound Healing

Mitoquinone slowed fibroblast migration across fresh wounds

Scratched primary human nasal epithelial cell (HNEC) air liquid interface (ALI) cultures took an average of 41.2 hours to re-appose (Fig. 1A1). The addition of mitoquinone did not affect ROS production or wound transit time ($p<0.05$) at any of the concentrations tested (Fig. 1B1).

Scratched fibroblast monolayers re-apposed significantly faster than HNEC, with untreated control cells closing after 27.1 hours, on average (Fig. 1A2). Both 10μM and 20μM of mitoquinone began significantly slowing fibroblast migration at 16 hours ($p<0.05$), and by 48 hours wounds treated with these doses had only closed <40% (Fig. 1A2). This effect was associated with a significant reduction in ROS from 24 hours at these two doses (Fig. 1B2). 5μM and 2μM of mitoquinone also significantly slowed fibroblast migration from 24 hours and 32 hours respectively ($p<0.05$), however, in those cases the effect was not associated with a discernible drop in ROS levels (Fig. 1B2). Final wound closure percentages averaged 31% for 5μM and 29% for 2μM after 48 hours. Doses of mitoquinone ≤1μM did not significantly alter wound transit times compared to negative controls, although there was a strong trend towards slower wound closure (48 hours vs. 27.1 hours on average, respectively).



Supplementary Figure 1: Standardised mechanical wound of HNEC **(A1, A2)** and fibroblast **(B1, B2)** monolayer 12 hours post wounding. Lines represent wound edge and arrows represent wound diameter. **(A1, B1)** wounded cell monolayers with no exposure to antioxidants, showing increased production of ROS (green), especially at the wound edge. **(A2, B2)** wounded cell monolayers treated with 5μM mitoquinone.

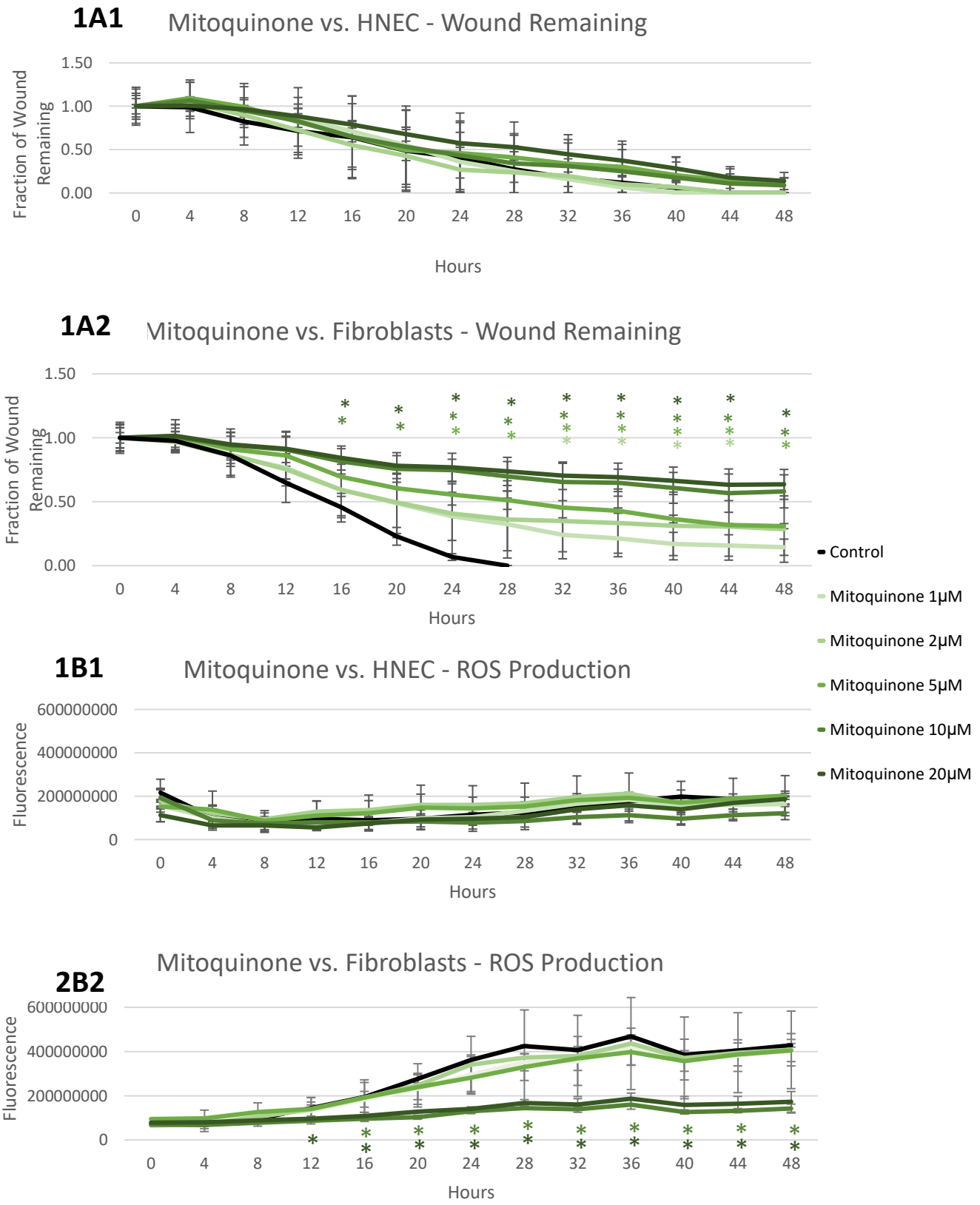


Figure 1: Effect of Mitoquinone on wound closure (1A1 and 1A2) and ROS production (1B1 and 1B2) of primary human nasal epithelial cell air liquid interface (HNEC-ALI) cultures (1A1, 1B1) and primary fibroblast monolayers (1A2, 1B2)

(A) Fraction of wound remaining of wounded HNEC_ALI and fibroblast monolayers (y-axis), normalized to wound at time=0 over 48 hours of continuous exposure to various dilutions of mitoquinone (shades of green) or control (cell media only, black).
(B) Levels of fluorescence emitted (y-axis) by wounded HNEC_ALI and fibroblast monolayers over 48 hours with exposure to various dilutions of mitoquinone (shades of green) or control (media only, black). Values represent fluorescence produced (arbitrary units) compared to unscratched, untreated cells interrogated under the same conditions.
* $p < 0.05$, Dunnett *post hoc* test vs. control (i.e. cell media only), for same cell type. Bars represent +\- SEM.

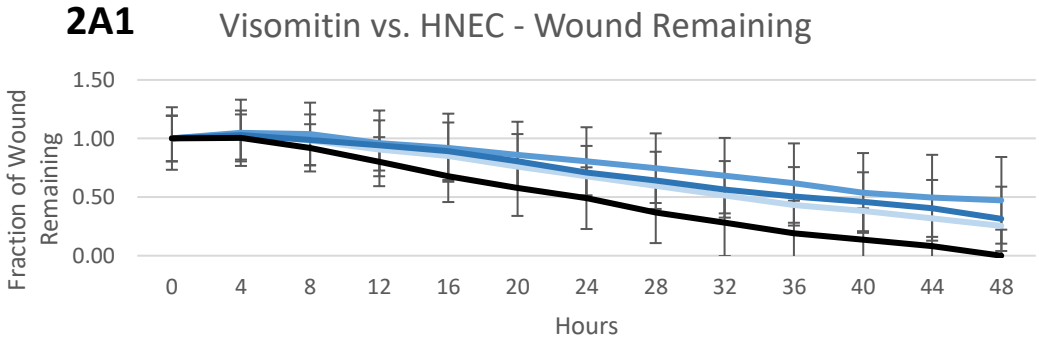
Visomitin slowed fibroblast migration across fresh wounds

As with mitoquinone, the addition of visomitin did not significantly affect HNEC ROS production or wound transit time ($p<0.05$) at any of the concentrations tested (Fig. 2A1, 2B1).

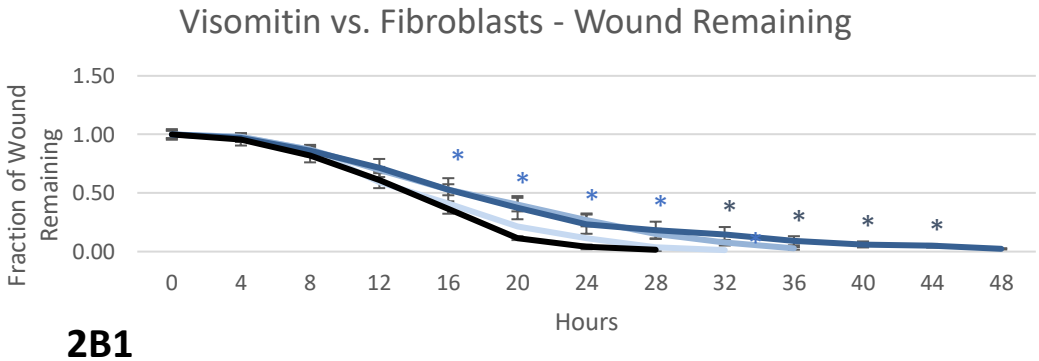
Doses of visomitin ≤1μM did not significantly alter wound transit times compared to negative controls. 5μM and 2μM of mitoquinone significantly slowed fibroblast migration from 16 hours and 32 hours respectively ($p<0.05$), however at these doses there was again no significant associated drop in ROS levels (Fig. 1B2.2). Unlike mitoquinone, all fibroblast wounds treated with 1-5μM closed within 48 hours.

Doses ≥ 10μM of visomitin were not tested, as these concentrations had already been found to be toxic to both cell types at the time of testing (see safety data below).

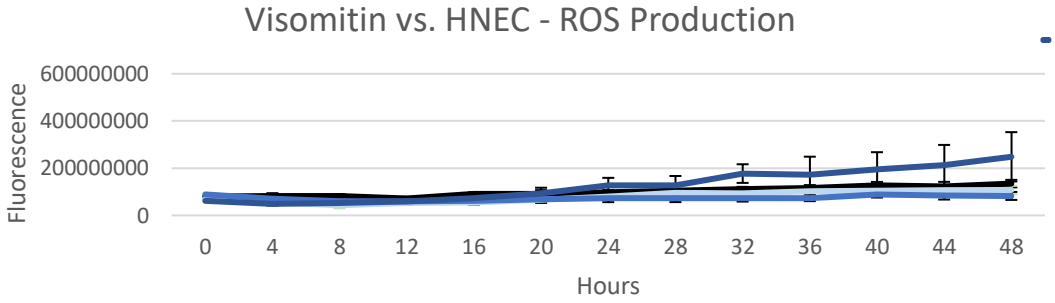
2A1



2A2



2B1



2B2

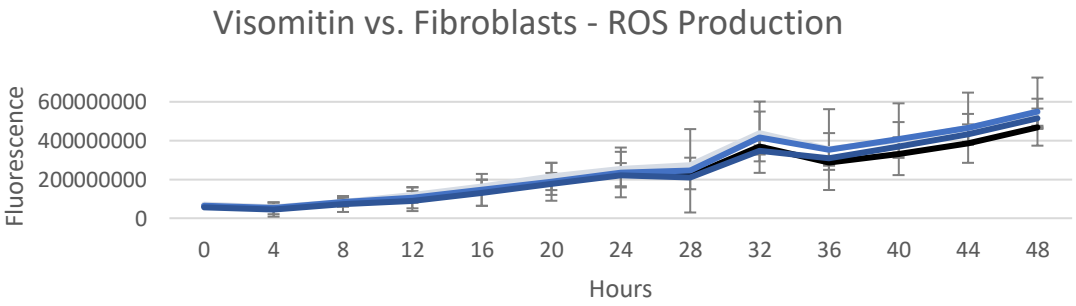


Figure 2: Effect of Visomitin on wound closure (2A1 and 2A2) and ROS production (2B1 and 2B2) of primary human nasal epithelial cell air liquid interface (HNEC-ALI) cultures (2A1, 2B1) and primary fibroblast monolayers (2A2, 2B2)

(A) Fraction of wound remaining of wounded HNEC_ALI and fibroblast monolayers (y-axis), normalized to wound at time=0 over 48 hours of continuous exposure to various dilutions of visomitin (shades of blue) or control (cell media only, black).
(B) Levels of fluorescence emitted (y-axis) by wounded HNEC_ALI and fibroblast monolayers over 48 hours with exposure to various dilutions of visomitin (shades of blue) or control (media only, black). Values represent fluorescence produced (arbitrary units) compared to unscratched, untreated cells interrogated under the same conditions.
* $p < 0.05$, Dunnett *post hoc* test vs. control (i.e. cell media only), for same cell type. Bars represent +\- SEM.

Safety Studies

Low dose MTAs did not induce cytotoxicity

An LDH cytotoxicity assay determined that mitoquinone was not cytotoxic to primary HNEC at doses ≤10 μM or primary human sinonasal fibroblasts at doses ≤5 μM following 48 hours of treatment exposure.

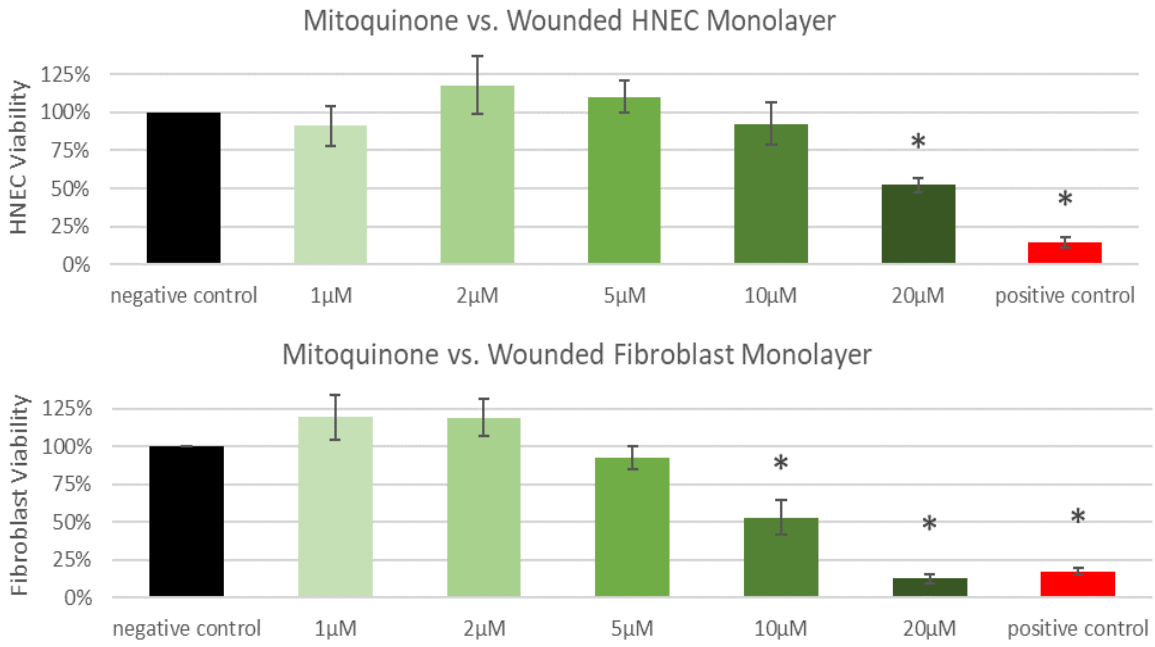


Figure 3: Primary HNEC and human fibroblasts cell viability after 48 hours exposure to mitoquinone, compared with cell media alone (negative control) and 2% Triton ×100 (positive control). Cell viability (y-axis) calculated using the inverse of % LDH release compared with negative control.
* $p < 0.05$, Students t-test vs. negative control.
Bars represent +\- SEM (3 patients with 2 technical replicates).

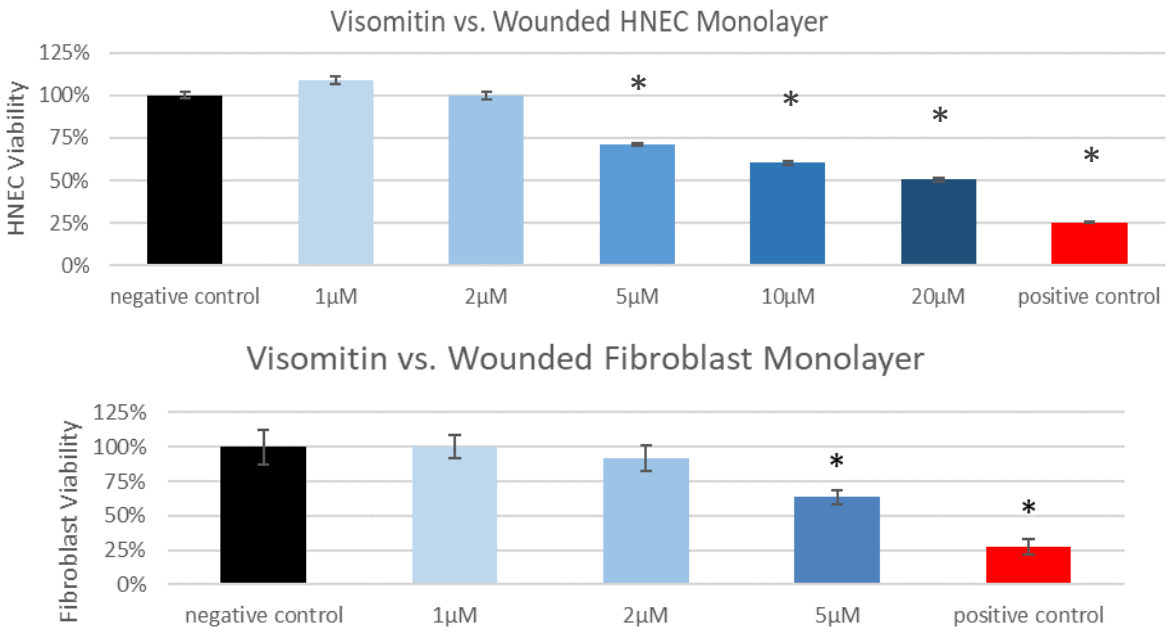
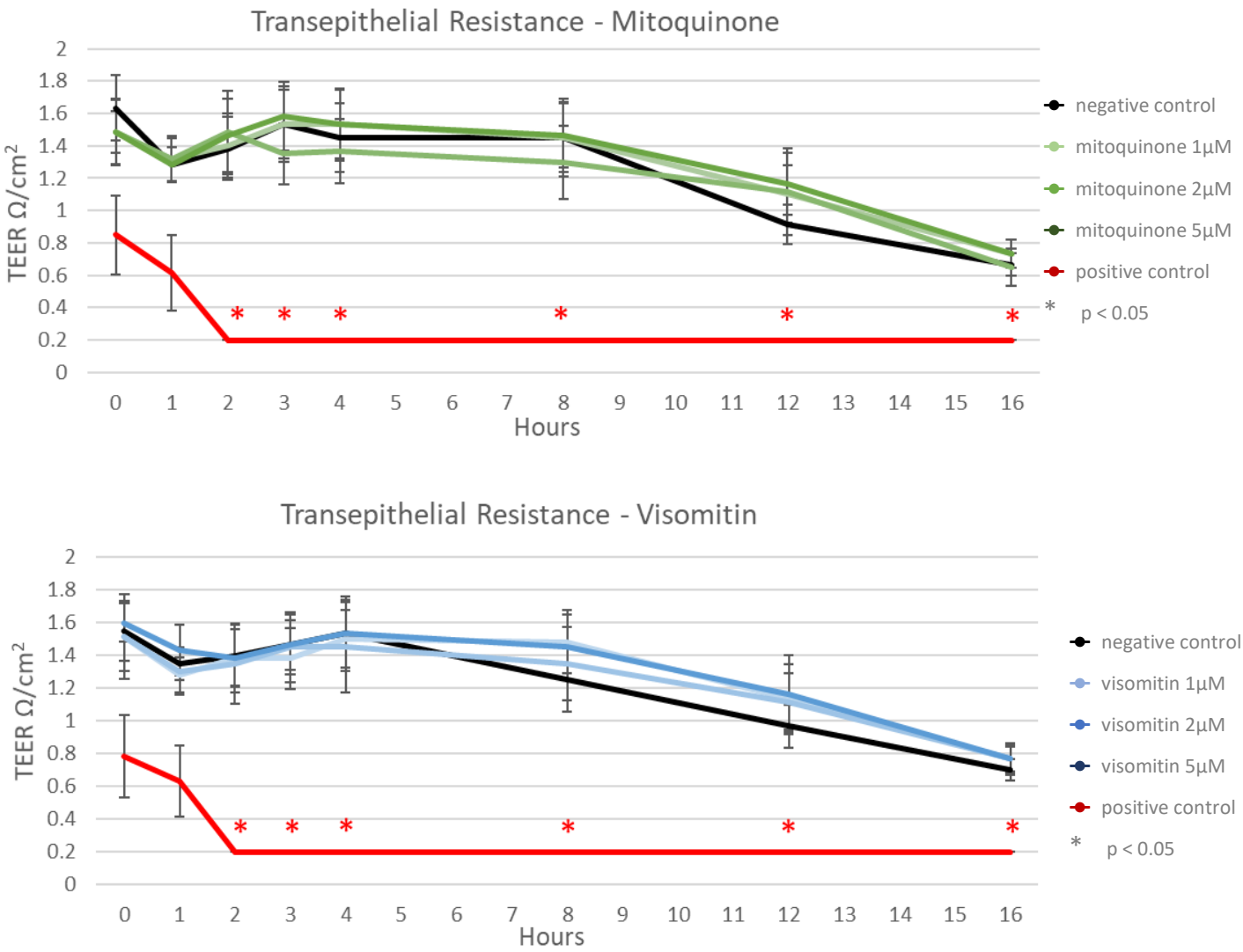


Figure 4 Primary HNEC and human fibroblasts cell viability after 48 hours exposure to visomitin, compared with cell media alone (negative control) and 2% Triton ×100 (positive control). Cell viability (y-axis) calculated using the inverse of % LDH release compared with negative control.
* $p < 0.05$, Students t-test vs. negative control.
Bars represent +\- SEM (3 patients with 2 technical replicates).

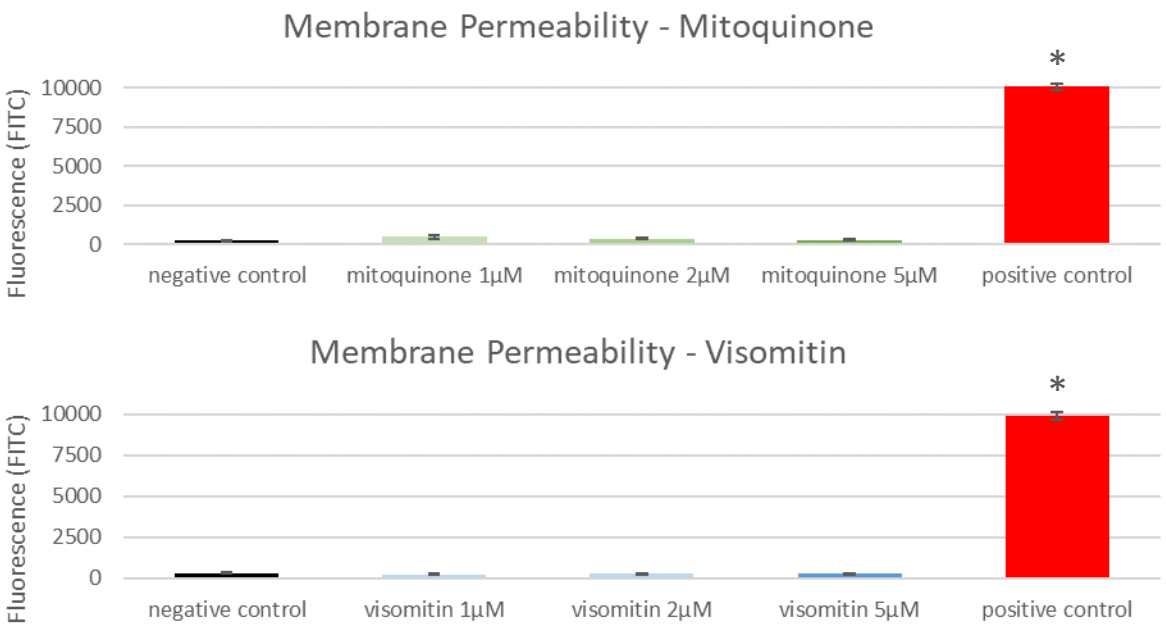
Visomitin began showing significant toxicity against HNEC at a lower concentration than that of mitoquinone (≥5 µM vs, ≥20 µM). However, both MTAs had similar toxicity against primary human sinonasal fibroblasts, with visomitin showing no evidence of cytotoxicity at doses ≤2µM following 48 hours of treatment exposure. As doses ≥ 10 µM had already been shown to be toxic to epithelial cells both macroscopically and functionally, these doses were not tested against fibroblasts.

Both MTAs did not alter HNEC trans-epithelial electrical resistance or membrane permeability at non-toxic doses

Both mitoquinone and visomitin did not alter trans-epithelial resistance in mature (> 8 weeks at air liquid interface culture), polarized, primary HNEC over 16 hours of topical submersion ≤ 5μM, compared to vehicle control (PBS alone), A FITC-dextran permeability assay was then used to confirm membrane integrity, and no significant difference was seen between either treatment (1-5 μM) and negative control:



Supplementary Figure 3: Primary human nasal epithelial cells trans-epithelial electrical resistance after 16 hours exposure to topical MTA, compared with cell media alone (negative control). Positive control = 2% Triton ×100. Time=0 is time immediately after application of control solution or test compounds
3 cell lines, 2 technical replicates each. * $p < 0.05$, t-test vs. negative control.
Bars represent +\- SEM (3 patients with 3 technical replicates).



Supplementary Figure 4: Fluorescence of media in basal chamber 2 hours after loading fluorescent FITC-dextran into apical chamber. This data represents primary human nasal epithelial cells membrane permeability after 16 hours exposure to each MTA, compared with cell media alone (negative control). Positive control = 2% Triton ×100. 3 cell lines, 2 technical replicates each. * $p < 0.05$, t-test vs. negative control. Bars represent +\- SEM (3 patients with 3 technical replicates).

Low dose mitoquinone did not alter HNEC cilia beat frequency, although visomitin did so after 12 hours exposure

Mitoquinone did not significantly alter cilia beat frequency (CBF) in mature (> 8 weeks at air liquid interface culture), polarized, primary HNEC over 24 hours of topical submersion ≤ 5μM, compared to PBS vehicle alone (negative control) (Figure 5). At 36 hours, only 5μM mitoquinone had significantly slowed CBF compared to negative control (56%, $p < 0.05$)).

Visomitin significantly slowed CBF beyond 4 hours at all three doses (1μM, 2μM and 5μM) ($p<0.05$). After 36 hours, mean normalized CBF for 1μM, 2μM and 5μM were 62%, 58% and 51% of negative control, respectively.

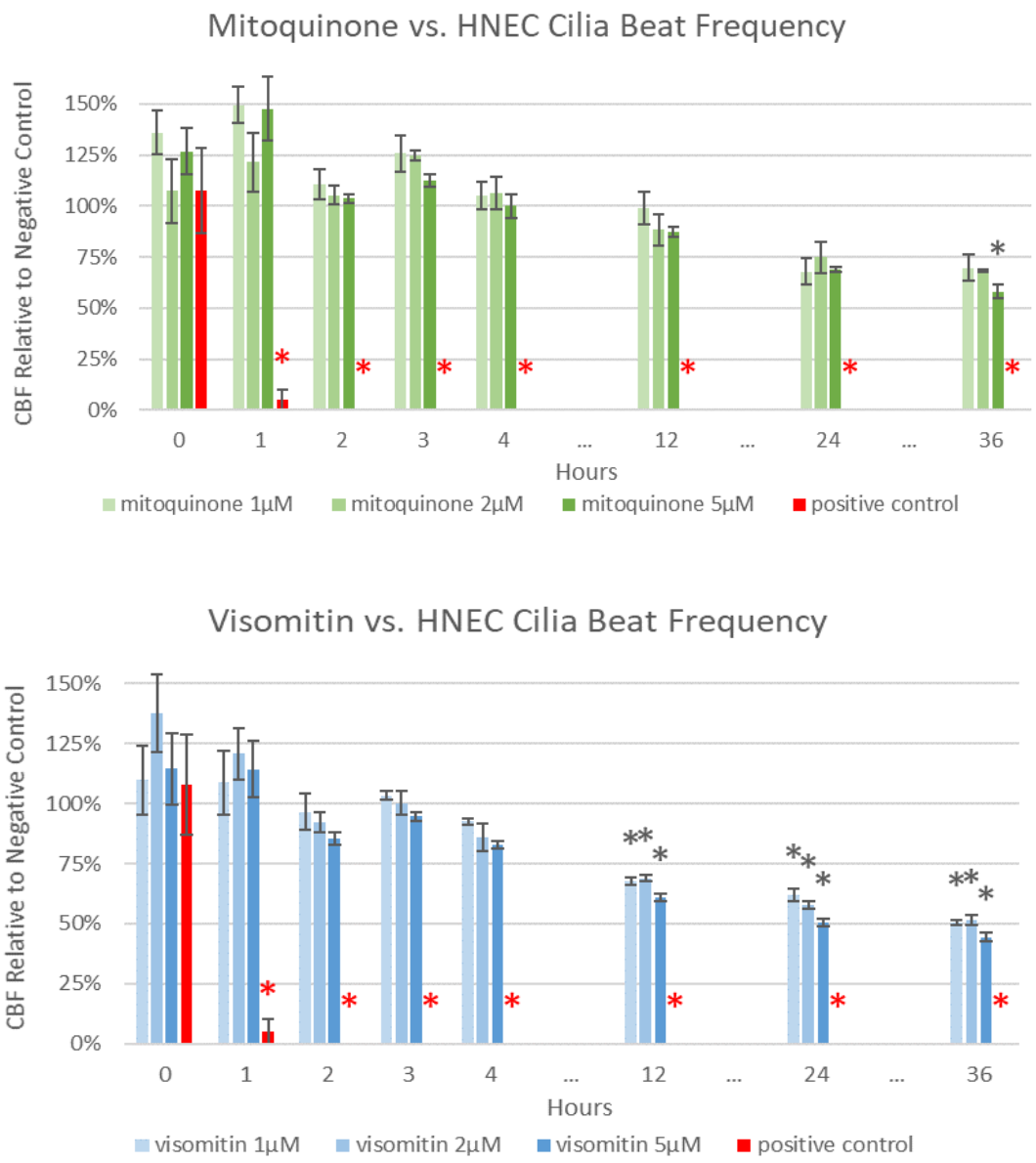


Figure 5: Primary human nasal epithelial cells air liquid interface cultures cilia beat frequency (CBF) over 36 hours exposure to topical mitoquinone and visomitin, normalized to CBF after application of PBS alone. Positive control = 2% Triton ×100. Time=0 is time immediately after application of control solution or test compounds
Cells from 3 independent donors, 2 technical replicates each. * $p < 0.05$, t-test vs. negative control. Bars represent +\- SEM.

DISCUSSION

Here we observed that the MTAs mitoquinone and visomitin both slowed fibroblast migration across freshly wounded cell monolayers. This was not associated with a slowing of epithelial cell migration, or toxicity at lower doses (2-5μM). This effect was significantly associated with a reduction in ROS levels at higher doses of mitoquinone (10-20μM) and this reduction was also associated with increased cytotoxicity at these concentrations, potentially due to a loss of ROS cellular housekeeping functions.

In vivo, this decrease in migration might limit the recruitment of fibroblasts and fibroblastic collagen deposition within the wound without compromising its epithelialization, leading to a lower risk of a fibrin bridge forming between two wounded surfaces. [164, 312] These properties may be beneficial in the post-surgical setting where normal epithelialization is required, but a reduction in fibroblast migration will be beneficial in reducing scar tissue or adhesion formation.

As previously stated, management of adhesions is usually assigned a high priority during revision surgery. Despite this, it is important to acknowledge that not all adhesions require revision surgery, depending on their density and, perhaps most importantly, their position. In fact some are intentionally created between the middle turbinate and septum, in order to prevent lateralisation of the middle turbinate and collapse of the middle meatus. [129]

With respect to the highly potent activity on fibroblast migration observed in the present study, it was important to investigate any collateral cytotoxicity of MTAs towards sinonasal cells. The lack of cytotoxicity seen after 48 hours exposure at concentrations ≤ 5μM was supported by the healthy cell morphology and activity observed during confocal microscopy. At ≤ 5μM, mitoquinone was also found to be non-toxic to primary human nasal epithelial cells grown at air-liquid interface; providing a high-fidelity model of its *in vivo* safety in terms of cell viability, cilia beat frequency and cell layer integrity. Doses ≥ 5μM were found to be toxic to HNEC and fibroblasts respectively, potentially indicating

that the level of ROS suppression caused by these doses was critically detrimental to the normal function of the cell. [191, 251]

Mitoquinone has previously shown a robust safety profile with human tissue both *in vitro* [255] and during multiple phase II human trials. [246, 272, 274] This mirrors visomitin, which has previously not displayed significant toxicity to HeLa cells exposed to ≤ 5μM concentrations for 17 hours. [255] Composite membranes loaded with up to 80μM of mitoquinone have also been used in animal models without adverse effects, although the precise release profile of the mitoquinone was somewhat unclear. [321] As such there is good preliminary evidence to support safe use of MTAs *in vivo* at doses used in the present experiment.

Whilst these findings are currently translational in nature, the present study is a necessary step in the move towards *in vivo* experimentation utilizing wound healing models with a functioning immune system. This study also delineates the inherent properties of the cells from any observations *in vivo*. Given the positive safety and efficacy profile *in vitro,* MTAs such as mitoquinone warrant further investigation as perioperative adjuncts to improve wound healing, especially in settings such as the sinuses that are prone to adhesions.

CONCLUSION

Both mitoquinone and visomitin slowed migration of fibroblasts across wounded cell monolayers. Higher doses of mitoquinone were also associated with significant reductions in recordable ROS levels, as well as cytotoxicity.

The effective, non-toxic dose range of both agents was approximately 2-5μM. This positive safety and efficacy profile, even at micromolar concentrations, means that MTAs hold great promise for improving wound healing outcomes in the postoperative setting.

Statement of Authorship

Title of Paper	Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Staphylococcal Infections in an Infected Cutaneous Wound Model
Publication Status	Prepared for Submission
Publication Details	Withheld Under Embargo

Principal Author

Name of Principal Author (Candidate)	Dr. Michael Gouzos		
Contribution to the Paper	Project design, animal ethics application, experimental work, data collection and analysis, manuscript preparation		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/12/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Project design, experimental work, data collection and analysis, manuscript preparation		
Signature		Date	7/12/20

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Contribution to the Paper	Project design, manuscript preparation		
Signature		Date	7/12/20

Safety and Efficacy of Mitochondrially-Targeted Antioxidants for Staphylococcal Infections in a Murine Infected Cutaneous Wound Model

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Figures: 5

Supplementary Figures: 2

Abstract

Introduction Impaired wound healing due to infection continues to present a challenging problem in the field of wound management. The emergence of multi-drug resistant organisms has led to increased interest in alternative solutions to antibiotics. The mitochondria-targeted antioxidant Mitoquinone has emerged as a novel therapy to treat bacterial infections. Here we investigate the safety and efficacy of Mitoquinone incorporated into a surgical hydrogel to treat acute *S. aureus* infected cutaneous wounds.

Methods 44 Balb/c mice were given standardized dorsal wounds and infected topically with a bioluminescent strain of *S. aureus* ATCC12600 (1×10^7 CFU/mL) carrying a modified *lux* operon from *Photorhabdus luminescens* (Xen29) (PerkinElmer, UK). Mature biofilms were allowed to form under the waterproof dressing for 48 hrs, followed by application of a mitoquinone infused hydrogel, hydrogel alone, or controls. Wound size was quantified every 2-3 days, while bioluminescence and colony-forming unit (CFU) assays were used concurrently to quantify infection severity. Wounds were harvested after 9 days, at which time bacterial load and histological wound size were measured. Finally, a safety profile for these treatments was sought against human epithelial cells.

Results Chitogel with 80µM mitoquinone showed a significant reduction in live bacteria within endpoint biofilms compared to infected controls and hydrogel alone. 20-80µM mitoquinone also reduced total wound area and wound length compared to positive controls and mupirocin. These concentrations were safe for use on human cells.

Conclusions Chitogel with mitoquinone shows potential as an antimicrobial agent which, unlike traditional antimicrobials, may also improve the speed and quality of wound healing.

Introduction

Wound infection, and the subsequent impaired healing of infected wounds, is a serious medical issue affecting thousands of people worldwide. Pathogenic bacteria that normally colonize the skin and surrounding environment can readily cause localised infections wherever the skin barrier is impaired. Some bacteria are also able to avoid attempts to eradicate them by forming clusters of bacteria enveloped in a protective polymeric substance, collectively termed a biofilm. [15, 16] The existing paradigm of treating these infections with antimicrobials has been hindered by, and indeed contributed towards, the emergence of multi-drug resistant (MDR) infections. These infections cause over 700,000 deaths/year globally and unless action is taken this number has been projected to reach 10 million deaths by 2050, with an economic cost of $USD100 trillion. [322] The development of new approaches to combat infections, that do not rely on antimicrobials, are needed to help regulate infections in patients with non-healing, chronic wounds.

Chitosan is one example of an alternate therapy that positively influences the healing process. A linear polysaccharide derived from the outer skeleton of shellfish, it exerts a positive effect on wound healing through the faster formation of granular tissue in the initial stages of healing, [323] and its derivatives also display antibiotic and antioxidant properties. [324] This antioxidant effect is significant, as increased oxidative stress and reactive oxygen species (ROS) within a wound have been shown to contribute to the chronicity of infection. [325, 326] Scavenging or preventing ROS formation is known to affect fibroblast migration and collagen deposition, [164, 279, 327] which may help prevent scar formation *in vivo*. [199-202, 279]

The inner mitochondrial matrix of cells is a major contributor to the production of ROS, and cells damaged by mitochondrial ROS become incapable of efficient repair. [328] For this reason, several mitochondrially-targeted antioxidants (MTAs) have been developed

specifically to treat mitochondrial oxidative stress, with mitoquinone emerging as one of the most widely studied. [247] Mitoquinone is an antioxidant quinone linked to a triphenylphosphonium (TPP) moiety by a 10-carbon alkyl chain, [247, 329] a structure that allows it to penetrate to the inner mitochondrial matrix far more efficiently than a standard antioxidant. Interestingly, this structure indirectly disrupts the membrane function of several bacteria, including *Staphylococcus aureus,* via a process termed protonophorous-like uncoupling. [255]

Mitoquinone has previously been added to composite membranes as a wound-healing adjunct in animal models of sterile wounds, where they displayed superior healing properties compared to control membranes. [321] However, crucially, its efficacy in infected wounds, which have the potential to develop into chronic ulcers or spread into systemic sepsis, remains unclear. Mitoquinone's effectiveness as an anti-biofilm treatment is also under investigation, given that this is a common and problematic bacterial adaptation to evade treatment and/or an immune response.

In the present study, we utilize a murine model of acute cutaneous injury to assess a mitoquinone-infused Chitosan hydrogel as a treatment for infected wounds. We hypothesized that it would have an antibacterial effect comparable to traditional antibiotics, whilst conveying an additional benefit to the speed and quality of wound healing.

Methods

Study Design

Mice were selected as an experimental model because they allow for a relatively high number of repetition and statistical certainty and they are genetically heterogenous.

44 mice (see 'animal studies' below) were examined using the following treatment groups:

- Two concentrations of mitoquinone in a fixed amount of Chitogel (2 groups x 8 mice)
- Chitogel alone (8 mice)
- Mupirocin topical ointment (as a 'gold standard' antimicrobial for *S. aureus,* 8 mice*)*
- Untreated, infected controls ('positive control', 8 mice)
- Untreated, uninfected controls ('negative control', 4 mice)

Treatments

Preparation of treatments

Mitoquinone mesylate (MedKoo Biosciences Inc., South Carolina, USA) was initially prepared as high concentration stock (3mg/mL) in 1:1 ethanol: milliQ water as per the manufacturer's instructions.

Chitogel was prepared 24hrs prior to use using a combination of three components; 5% succinyl-chitosan, phosphate buffered saline and 300mg dextran aldehyde (Chitogel®, Wellington, NZ). The components were manufactured and sterilized by Chitogel®. Mitoquinone was added to the phosphate buffer at the desired concentration just prior to mixing. The gels were then allowed to set for 24hrs at 4 degrees Celsius. An 8mm biopsy punch was used just prior to treatment application to excise a circular portion of gel that could be placed within the cutaneous wound.

An anti-*staphylococcal* pseudomonic acid antibiotic already used in clinical practice, mupirocin, was used as a 'gold standard' antimicrobial comparison control. A syringe was used to apply 0.05mL of 2% w/w mupirocin cream (GlaxoSmithKline Australia, Victoria, Australia) in an identical manner to gel treatments.

***In Vitro* Experiments**

Mitoquinone gel release

Prior to use on animals, the release profile of mitoquinone from Chitogel was assessed. A target concentration of 1-5µM was selected, as this was the effective range of mitoquinone against *S. aureus* seen in previous studies. [255, 327] Treatments were prepared as above, and then 10mL of gel was submerged in 10mL PBS in a 30mL glass cylindrical container. Containers were incubated at 37°C on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. A 100 µL sample of PBS was taken every 24hrs for 7 days and replaced with fresh PBS.

Mitoquinone gel release quantification assay by HPLC-MS/MS

Samples were evaluated using high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). This assay was validated using an "in-house" calibrator and quality control (QC) material. Briefly, each calibrator batch was prepared in PBS by adding a fixed amount of mitoquinone stock (20 µg/L) to make 5-0.05 µg/L calibrators. QC samples were prepared using a separate mitoquinone stock solution (20 µg/L) flask to make 2.00, 0.50 and 0.20 µg/L calibrators in PBS. Precipitation solution was prepared by adding 1000 µL of 20 µg/L Cetrimide internal standard stock solution to a 10mL volumetric flask with 9mL methanol.

Samples were prepared by aliquoting 50 µL of calibrators, QCs, or sample in 1.5 mL Eppendorf tubes, with care taken to disperse the QC specimens evenly throughout the run. Precipitating solution (300 µL) was then added to all samples and vortex mixed.

Tubes were then centrifuged for 5 mins at 13200 rpm to remove any gel or precipitants and 150 µL of supernatant was transferred to HPLC tubes for analysis.

For analysis, 20 µl of the sample was injected into the HPLC system (AB-Sciex UHPLC system with AB-Sciex Tandem Mass Spectrometer API-4500 and a Supercosil LC-18, 3.3 cm × 4.6 mm 3 µm column; Flow program 50% 2mM Ammonium Acetate in Water [A] 50% 2mM Ammonium Acetate in Methanol for 1.5 mins increasing B to 95% at 2.5 min and held for 2.5 mins, then returning to initial conditions at 6 mins). Mitoquinone was detected using positive electrospray ionization with multiple reaction monitoring (583.3 – 441.2 Da) and quantified using the peak area ratio of mitoquinone to the cetrimide (338.3 – 55.0 Da) peak.

Calibration curves were linear ($r^2 > 0.9949$). Intra- and inter-assay inaccuracy and imprecision were < 10% at the lower limit of quantitation and < 5% at the upper limit of quantitation. No matrix effects were observed. Runs were accepted if the QC results were within 2xSD of the target values (0.20, 0.50 & 2.00 µg/L). Results are expressed in µM for each sampling timepoint.

Animal Studies

This study was performed in accordance with guidelines approved by the Animal Ethics Committee of the University of Adelaide (reference AECM03/20). 44 Balb/c mice (50% male, 50% female, 10-12 weeks old) were housed in a sterile PC2 facility and allowed to acclimatize for one week with access to *ad libitum* food and water. Following wounding surgery, animals were weighed daily as a surrogate measure of constitutional stress, and their wellbeing was monitored using a validated, 9-item welfare score incorporating signs and symptoms of systemic bacterial sepsis (reluctance to move, sunken orbits etc.)

Mouse Wounding and Bacterial Inoculation

On the first day of the experiment, all mice were anaesthetised with inhaled anaesthetic isoflurane and a dose of subcutaneous buprenorphine (0.05mg/kg) was administered for

analgesia. The backs of the mice were shaved using clippers and depilated using Veet™ Cream ('Sensitive', Reckitt Benckiser, Slough, United Kingdom). The cream was then removed and the skin sterilised using alcohol and chlorhexidine tinctures. A 10mm biopsy punch was used to make a single, standardized circular wound on the dorsal aspect of each mouse. Wounds were then immediately photographed as a baseline measurement for the wound quantification studies, using a ruler for scale.

Wound infections were then created with a genetically modified strain of bioluminescent *S. aureus* ATCC12600 carrying a modified lux operon from *Photorhabdus luminescens* (Xen29) (PerkinElmer, UK). This allowed quantification of the infection using luminescent imaging. Glycerol stocks of Xen 29 were grown on nutrient agar for 48 hours, and then transferred to broth for a further 24 hours. Prior to use this broth was titrated to a 1x10^7 CFU/mL solution. Xen 29 is resistant to Kanamycin (Sigma Aldrich, Steinheim, Germany), allowing for selective growth of Xen 29 on 200 µg/mL Kanamycin nutrient agar during all subsequent assays, to minimise any chance of contamination with non-Xen 29 bacteria.

For inoculation, a 100 µL pipette was used to dose the center of the wound with 40 µL of fresh suspension of bioluminescent *S. aureus* in PBS, at a concentration of 1x10^7 CFU/ml. All wounds, including negative controls, were then covered with a waterproof dressing (Tegaderm™, 3M, Minnesota, United States) to prevent leakage of bacteria, slow the dehydration of the wound surface and allow biofilms to form.

The level of *S. aureus* infection was quantified with bioluminescent imaging on an IVIS Lumina XRMS camera (Perkin Elmer, Waltham, USA) on days 2, 6 and 9 following inoculation. The region of interest was uniform for each mouse and each timepoint. Results were expressed as total flux (photons/s). In order to maximize image quality, bandages were removed prior to imaging, which also necessitated re-application of treatment on day 6.

Treatment Applications

48hrs after surgery, the mice in all arms were anaesthetised with isoflurane and randomized to receive standard of care (topical mupirocin cream), concentrations of mitoquinone in Chitogel applied topically (0 µM, 20 µM and 80 µM), or left untreated. Treatments were refreshed on day 6, as stated above.

Bandage CFUs

Identical-sized waterproof dressings (3x3cm Tegaderm™, 3M, Minnesota, United States) were used to cover all wounds at the point of initial inoculation and changed at each treatment. When these dressing changes occurred, the dressings were immediately immersed, *in toto,* in 400µL PBS in an Eppendorf tube and placed on ice. These tubes were then sonicated, immersed in ice, for 15 minutes using a 250TD ultrasonic cleaner (SoniClean, Adelaide, South Australia, Australia). The PBS was then assessed for colony forming units (CFUs) of *S. aureus* using a standard assay. [330] Briefly, PBS samples were serially diluted and 10µL plated onto *S. aureus* selective (200 µg/mL Kanamycin-impregnated) nutrient agar in triplicate. These plates were then incubated for a further 24 hours at 37 degrees Celsius in air and read the following day. Numbers of colonies were counted and averaged across the three replicates for each treatment. The number of CFU/mL was then derived from the dilution factor.

Macroscopic Wound Analysis

Macroscopic images of the wound were measured using Image Pro Plus v7.0.1 Software (Media Cybernetics Inc. Maryland, USA). Image distances were first calibrated using a ruler included in the original image, and then wound size (area in mm^2) and wound gape (horizontal distance across middle of the wound) were quantified separately for each of the standard timepoints (day 0, day 2, day 6 and day 9). Results are expressed as average measurement ± standard error of the mean (SEM).

Wound CFUs

At endpoint, the wounds were carefully excised and divided into three pieces (25%, 25% and 50%) for CFU analysis, biofilm analysis and histology respectively. The wound segment reserved for CFU analysis was immediately immersed in 200µL PBS into a pre-weighed Eppendorf tube. These tubes and their contents were then sonicated, immersed in ice, for 15 minutes using a 250TD ultrasonic cleaner (SoniClean, Adelaide, South Australia) to dislodge planktonic bacteria. The media was then interrogated for CFUs of *S. aureus*. As with bandage CFU analysis, samples were serially diluted and plated onto selective, Kanamycin-impregnated nutrient agar in triplicate. These plates were then incubated for a further 24 hours at 37 degrees Celsius in air and read the following day. Numbers of colonies were counted and averaged across the three replicates for each treatment. These concentrations were then individually scaled for wound weight, giving the number of CFU/mg of wound.

Biofilm Analysis

S. aureus wound biofilms were also qualitatively assessed using BacLight staining and confocal microscopy. For biofilm analysis, excised wounds were immediately immersed in Eppendorf tubes containing DMEM (Gibco, Massachusetts, USA) and placed on ice. Tubes were then sonicated, immersed in ice, for 15 minutes using a 250TD ultrasonic cleaner (SoniClean, Adelaide, South Australia) to dislodge planktonic bacteria, after which time the biofilms were carefully washed twice with PBS to remove these planktonic bacteria. Once dry, biofilms were loaded onto slides and dyed with 1 mL of MilliQ water containing 1.5 µL each of LIVE/DEAD BacLight stains SYTO9 and propidium iodide (Invitrogen Molecular Probes, Mulgrave, Australia). Slides were then incubated in the dark at 21^0C for 15 minutes. Biofilms were given a final wash with PBS to remove excess dye, fixed with cover slides and observed in a Zeiss LSM700 confocal LASER microscope (Zeiss Microscopy, Germany) at 10x magnification using two channels of a

split beam: λexcitation = 488 nm/λemission = 518 nm (Live bacteria, represented as green) and λexcitation = 555 nm/λemission = 585 nm (dead bacteria, represented as red). A ‘z-stack’ of 20 images, at 1 µm intervals, was compiled at three separate points for each biofilm using Zen Black software (Zeiss Microscopy, Germany).

Biofilms were quantified using Comstat 2.1 (Comstat, Kongens Lyngby, Denmark) using a validated protocol. [331, 332] The analysis included connected volume filtering, biomass analysis, thickness distribution and thresholding. Results are expressed as live biomass ($\mu m^3/\mu m^2$).

Histologic Wound Analysis

The remaining wound sections were loaded into cassettes and fixed in 10% neutral buffered formalin overnight before histological tissue processing and paraffin-embedding. The following day they were cut into duplicate 5µM sections and stained using either Lille-Mayer’s Haematoxylin/Eosin or Masson’s Trichrome. These stained sections were imaged using light microscopy at 10x. Image Pro Plus v7.0.1 Software (Media Cybernetics Inc. Maryland, USA) was then used to take the following measurements:

- wound length: the distance between the two pre-existing epithelial edges at the most central portion of the wound (i.e. diameter)
- dermal gape: the distance between the two pre-existing dermal edges at the most central portion of the wound
- percentage re-epithelialisation: the length of the newly epithelialized sections on either edge of the wound, divided by total wound length
- epithelial thickness: the depth of the newly formed epithelium on either side of the wound

Results are expressed as average measurement ± standard error of the mean (SEM).

ImageJ Software (v1.52a, National Institutes of Health, USA) was used to quantify collagen staining (green signal) in the wound area of the Masson’s Trichrome-stained

histology slides, using the RGB histogram function. Results are expressed as average green signal ± standard error of the mean (SEM).

Human Studies

Study population

Human cell studies were performed in accordance with guidelines approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital and the University of Adelaide (reference HREC/15/TQEH/132). All patients that donated cells gave informed consent and all samples obtained were anonymised and coded before use. Primary human nasal epithelial cells (HNECs) were harvested from nasal mucosa using an established protocol. [285] Sinonasal cells were collected using a standard cyto-brush, while the patient was under anaesthesia for a surgical procedure. Exclusion criteria included active smoking, age less than 18 years, pregnancy, systemic immunosuppressive disease and underlying malignancy.

Harvesting and air-liquid interface culturing of human nasal epithelial cells *in vitro*

Cells were suspended in PneumaCult™-Ex Plus Medium (STEMCELL Technologies Australia, Tullamarine, Australia) and depleted of macrophages using anti-CD68 (Dako, Glostrup, Denmark) before being cultured with Ex Plus Medium in collagen coated flasks at 37°C with 5% CO_2. HNECs were grown until confluent and seeded onto collagen coated 6.5 mm permeable Transwell® plates (Corning Incorporated, Corning, USA) at a density of 5×10^4 cells per well. Cells were maintained with Ex Plus medium for 2 days in a cell incubator at 37°C with 5% CO_2. On day 3 after seeding, the apical media was removed and the basal media replaced with PneumaCult™-ALI differentiation media (STEMCELL Technologies Australia, Tullamarine, Australia), exposing the apical cell surface to the atmosphere. HNEC at air liquid interface (HNEC-ALI) were maintained for a minimum of 21 days prior to experimentation for development of tight junctions and ciliation. [286]

Cytotoxicity studies

HNECs were maintained in a fully humidified incubator with 5% CO_2 at 37°C prior to cytotoxicity studies. Cells exposed to graded micromolar concentrations of mitoquinone or control (medium only) for 48 hours, followed by determination of lactate dehydrogenase (LDH) with a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA). Briefly, 50 µL of the supernatant from each well was mixed with 50 µL of assay reagent and incubated for 30 minutes in the dark at room temperature. After addition of stop solution, the optical density (OD) was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) and compared across treatment and control groups. Cell media alone was used as the negative control, 2% Triton X100 solution was used as the positive control, and an LDH standard included with the detection kit was tested for quality assurance, as well as blank cell media. Cell viability was determined by comparing LDH release of the untreated control with that of treatment groups. Pursuant to International Organization for Standardization 10993-5, percentages of cell viability above 80% were considered non-cytotoxic. [333]

Statistics

The power calculation and results were reviewed by a qualified statistician (GB). Treatment groups in endpoint experiments were compared using 2-way ANOVA. For experiments with multiple sampling points, Dunnett's test was used to compare differences between groups. A p-value < 0.05 was considered significant.

Results

Safety Studies

Mitoquinone Release from Chitogel

Mitoquinone showed a relatively limited, logarithmic release profile from both concentrations of gel after 96hrs: 5.56 µM (7%) from 80 µM gel and 0.79µM (4%) from 20µM gel. This equates to 3.7 µg/mL and 0.53 µg/mL respectively. The release profile of the 80µM gel fell largely within the target concentration range of 1-5µM of mitoquinone over the first 48hrs i.e. the range within which it has an antimicrobial effect. [255]

Human cell toxicity Studies

Human nasal epithelial cells were grown at air-liquid interface and treated with three concentrations of Chitogel +/- mitoquinone (0µM, 20µM, 80µM). None of the treatments showed a significant reduction in human nasal epithelial cell viability compared to untreated controls (i.e. <80% of negative control), [333], indicating that they are safe treatment in this setting.

Mouse Weights/ Welfare

All 44 mice survived through to endpoint, and no mice required additional pain relief or humane euthanasia during the study, as indicated by daily welfare checks and scoring. Infected, untreated mice (positive controls) lost 1g of weight on average at the end of the 9-day study, while uninfected mice (negative controls) did not lose any weight, on average. There was no significant weight difference between any of the treatment groups.

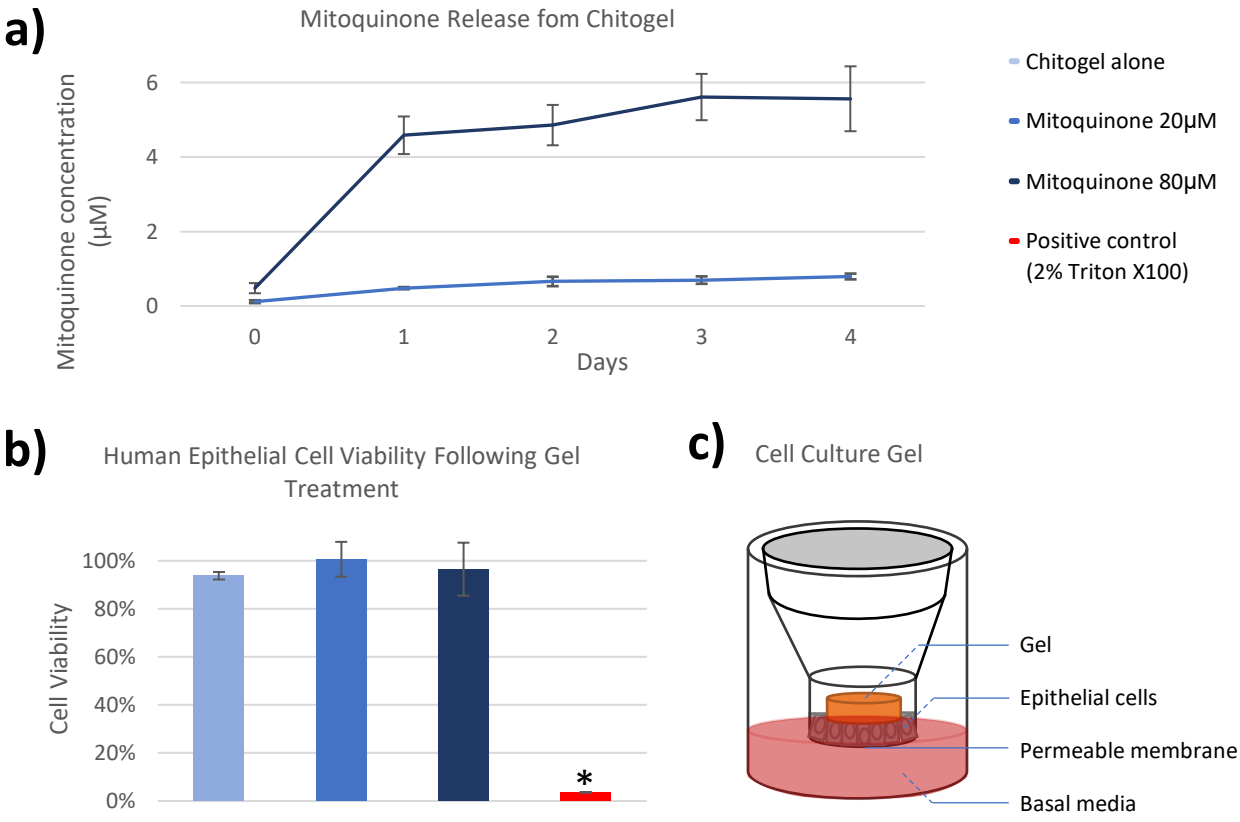
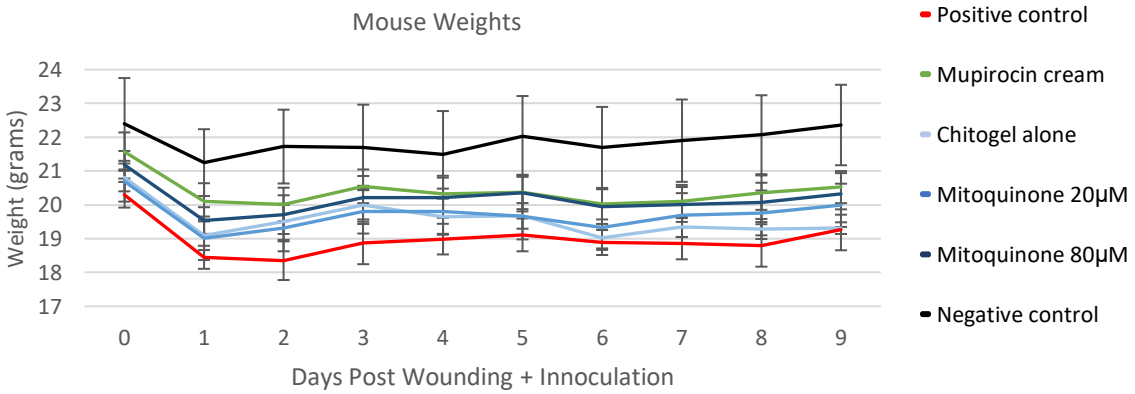


Figure 1a: Release profile of mitoquinone from mitoquinone-Chitogel. Concentration of mitoquinone (µM) in 10mL PBS overlying 10mL of mitoquinone-Chitogel with mitoquinone at 80µM and 20µM at 24hr intervals for up to 4 days. Bars represent SEM.
Figure 1b: Average cell viability for each treatment, compared to negative control (no gel). Values derived from relative release of LDH in basal media after 72hrs incubation. Bars represent SEM. *=p<0.05
Figure 1c: Schematic Corning Transwell® with mature human epithelial cells at air-liquid interface, covered by gel.



Supplementary Figure 1: Average weight of mice in each of the treatment groups on each day of the experiment. Bars represent SEM.

Animal Studies

Bioluminescent Imaging

Bacterial activity was measured by bioluminescence, prior to bandage application on day 0, day 2, day 6 and day 9. Mupirocin (a gold standard anti-*staphylococcal* control) showed a significant reduction in wound bioluminescence at day 6 ($p < 0.05$) and again at day 9 ($p < 0.05$) compared to positive control (Figure 2b and 2c). None of the other treatments showed a significant reduction compared to negative control. As expected, there was no bioluminescence detected on the negative controls.

CFUs in Wound

CFUs for each wound were derived at endpoint relative to the weight of the wound fragment (mg) and after immersion and sonication of the wound in PBS, relative to the volume of PBS (mL). No significant difference was seen between positive control and any of the treatment groups (Figure 2d). As expected, there was no growth detected on the negative controls.

CFUs on Removed Bandages

Bacterial load of the exudative material found on bandages was measured each time bandages were changed: prior to the initial treatment application on day 2, the treatment reapplication on day 6 and the day 9 endpoint. As expected, there was no growth detected on the negative controls. There was no significant difference in CFU counts between the bandages of any of the treatment groups, including the mupirocin control (Supplementary Figure 2).

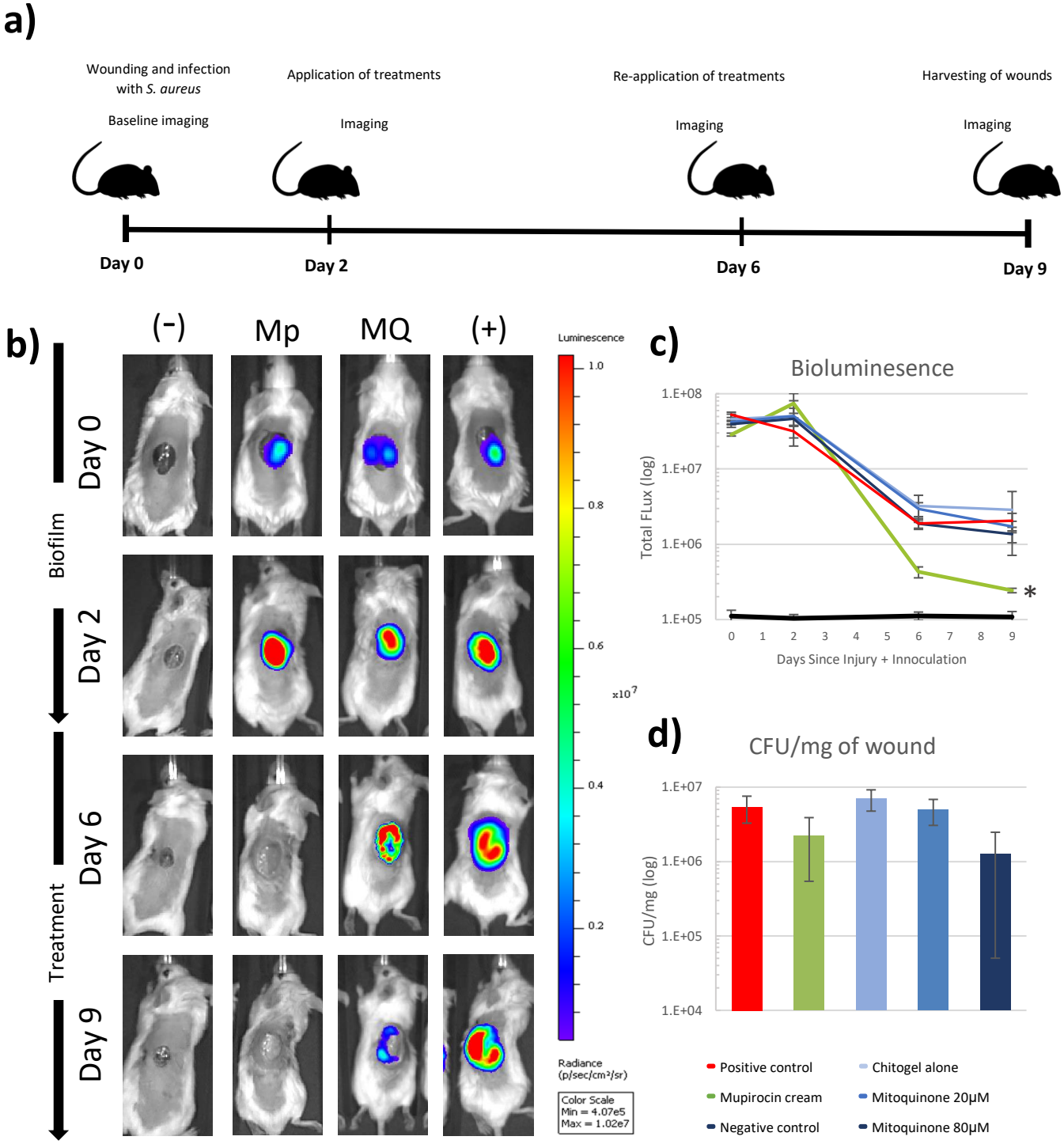
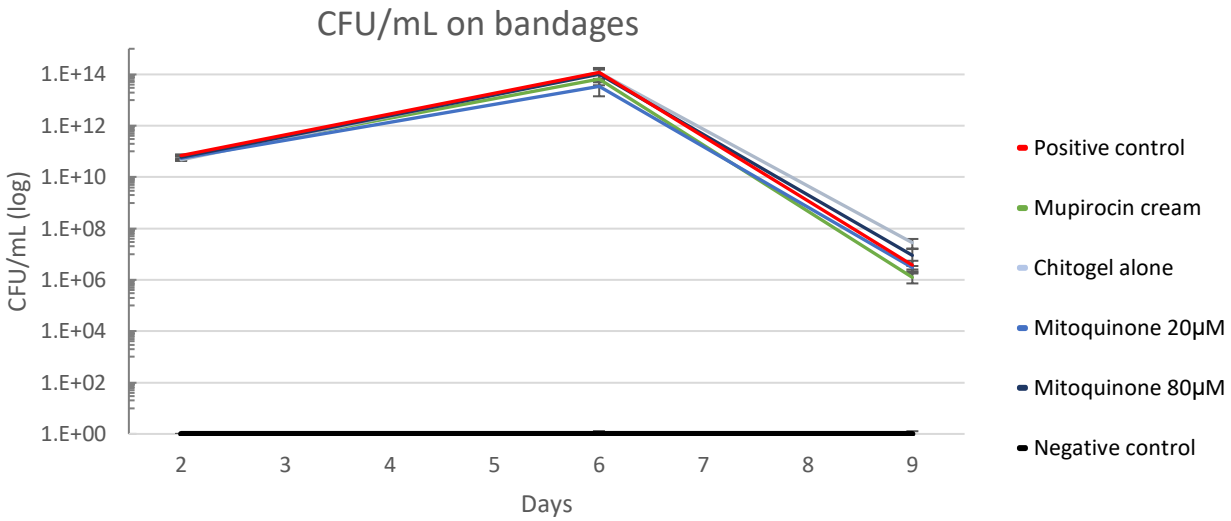


Figure 2a: Timeline of infection, treatment and imaging during the experiment
Figure 2b: Representative images of *S. aureus* biofilm wound bioluminescence (a surrogate for infection severity). Rainbow scale set across all images.
(-): negative control **Mp:** mupirocin control **MQ:** Chitogel with mitoquinone 80μM **(+):** positive control
Figure 2c: Average total flux (photons/s) for bioluminescent imaging of wounds from each of the treatment groups. Bars represent SEM. * = $p < 0.05$ t-test compared to positive control.
Figure 2d: Average CFU/mg on the wounds of each of the treatment groups at endpoint. Bars represent SEM. * = $p < 0.05$ Dunnett's test compared to positive control.



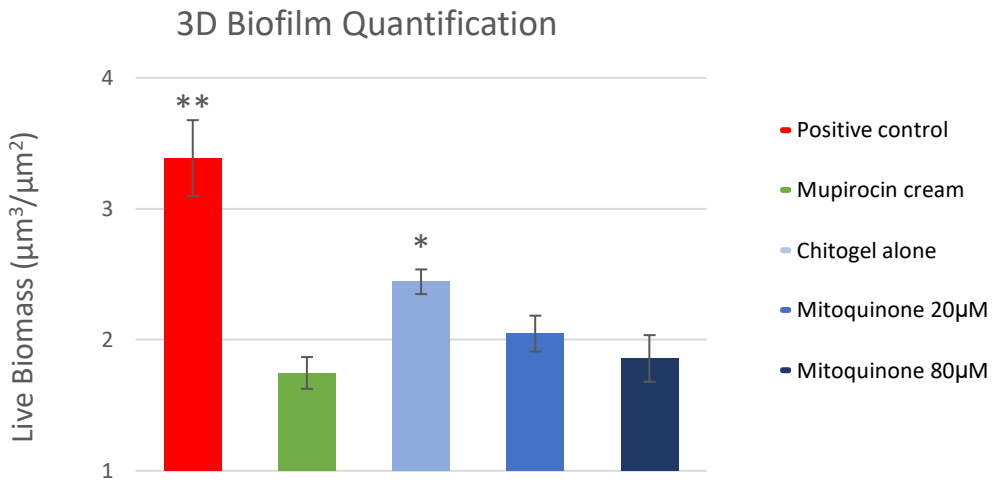
Supplementary Figure 2: Average bacterial load (in CFU/mL) removed on the bandages of each treatment group, at three separate timepoints (days 2, 6 and 9) Bars represent SEM.

3D Biofilm Analysis

No significant difference was observed between the negative control, mupirocin control and mitoquinone-Chitogel treatment groups (Figure 3a). Chitogel alone had significantly more live bacteria than negative controls ($p<0.05$). Positive controls had significantly more live bacteria than all other groups ($p<0.05$).

Qualitative analysis of the post-treatment biofilms showed predominantly dead bacteria (red) with a 'crust' of live bacteria (green), mainly on the wound peripheries (Figure 3b). Quantification of the live bacteria signal in each of these biofilm segments, using Comstat software (Kongens Lyngby, Denmark), yielded further information on the effect of these treatments.

a)



b)

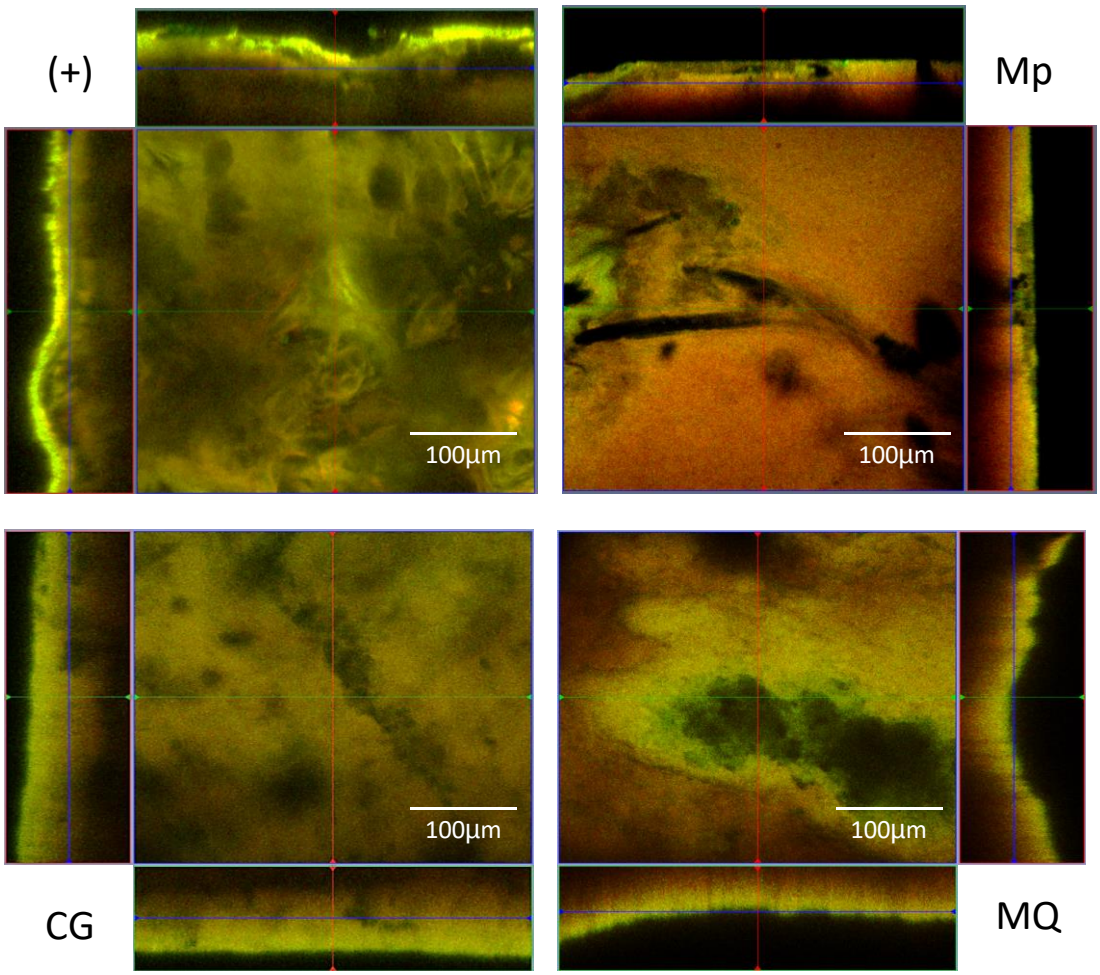


Figure 3a: Average live biomass (µm³/µm²) on the wounds of each of the treatment groups at endpoint. Biomass is calculated using Live/Dead Viability stain and Comstat biofilm quantification software. Bars represent SEM. * = $p < 0.05$ compared to negative control. ** = $p < 0.05$ compared to all other groups.
Figure 3b: Representative images of biofilm with live (green) and dead (red) staining, taken at endpoint **(+)** Positive control **Mp)** Mupirocin control **CG)** Chitogel alone **MQ)** Chitogel + Mitoquinone 80µM.

Macroscopic Wound Analysis

Uninfected (negative) controls showed a significant reduction in wound area and wound gape from day 6, compared to infected (positive) controls ($p < 0.05$), which tended to increase in size over time (Figure 4a and 4c). Chitogel with mitoquinone 80μM and 20 μM showed a significant reduction in wound area compared to positive control from day 6 and day 9 respectively ($p < 0.05$). No significant reduction in wound area was seen in either the mupirocin or Chitogel only groups compared to positive control.

Histologic Wound Analysis

Histologic analysis of the wounds was undertaken at endpoint (9 days after initial wounding and innoculation with bacteria). Control wounds showed an average wound length of 17.23 ± 0.64mm if infected and 6.95 ± 1.28mm if uninfected. Compared with infected (positive) controls, Chitogel with 80μM of mitoquinone significantly reduced wound length to 14.11 ± 0.97mm ($p<0.05$), equating to a -18.1% difference, on average (Figure 4b and 4e). This significant reduction was not seen in any of the other treatments, including mupirocin. However, there was a reductive trend in Chitogel (14.75 ± 0.81mm) and Chitogel with a lower dose (20μM) of mitoquinone (14.42 ± 1.28mm). Both dermal gape and wound re-epithelialisation were not significantly altered by any treatment, compared to positive controls (Figure 4G and 4H). Epidermal thickness was not significantly altered by any treatment, nor by the absence of infection (Figure 4F).

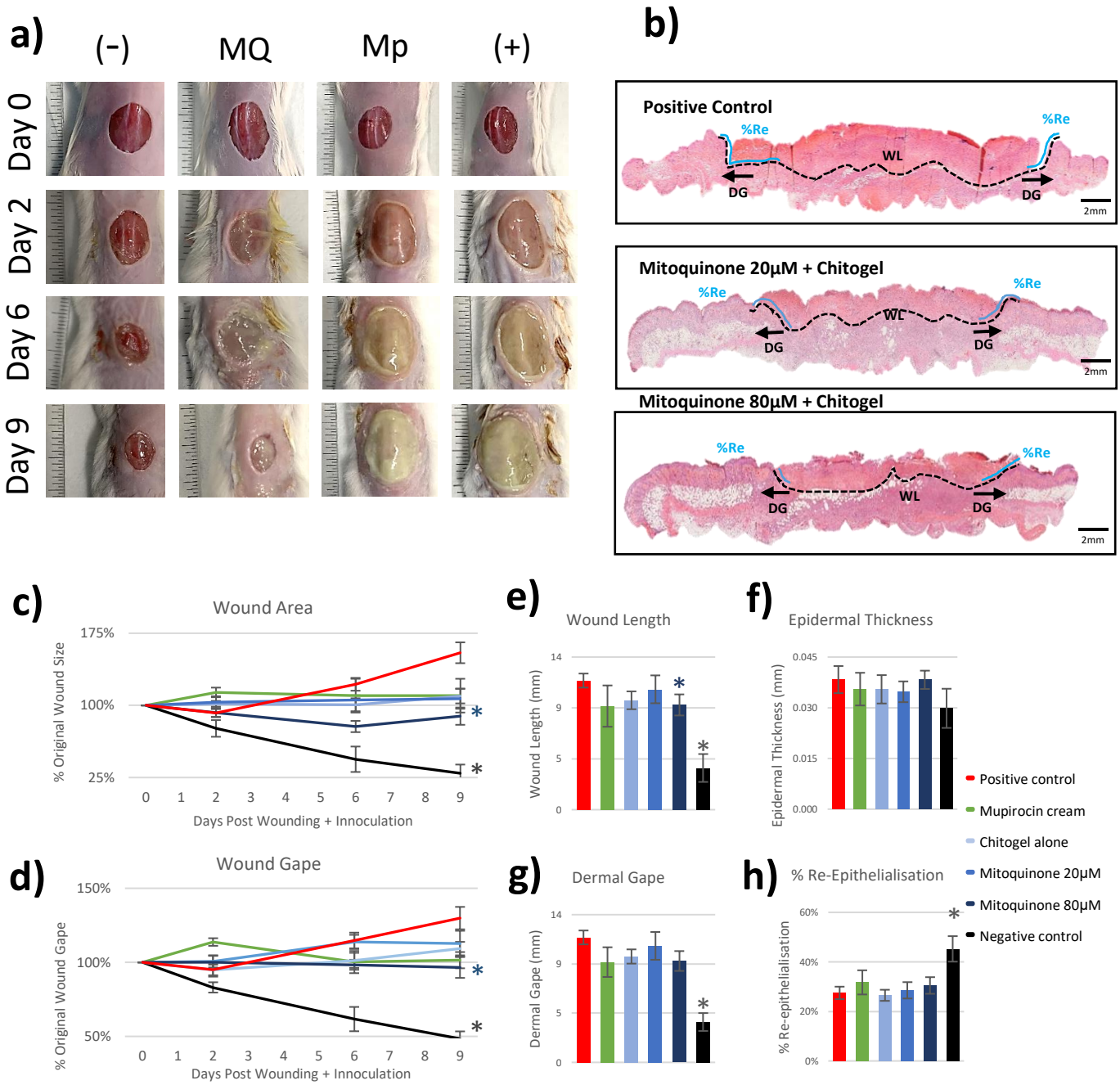


Figure 4a: Representative images of mouse wounds at four progressive timepoints **(-):** negative control **Mp:** mupirocin control **MQ:** Chitogel with mitoquinone 80µM **(+):** positive control

4b: Representative histologic sections of dorsal wounds, taken at end point with H&E staining for negative control, mitoquinone 80µM in Chitogel and positive control. Black dotted lines marks remaining wound length (WL), blue solid lines mark re-epithelialisation (%Re) and arrows mark the edge of dermal gape (DG).

4c: Average percentage change is wound area and **d)** wound gape for each of the treatment groups and controls. Bars represent SEM. * = $p < 0.05$ Dunnett test compared to positive control.

4e: Average histologic measurements of dorsal wounds taken at end point for wound length, **f)** epidermal thickness, **g)** dermal gape and **h)** percentage of wound covered in neoepithelium (i.e. % re-epithelialisation). Bars represent SEM. *=$p<0.05$ t.test vs. positive control.

Masson's Trichrome staining was undertaken at endpoint to determine the effect of the experimental conditions and treatments on wound collagen deposition. All treatment groups showed significantly higher average green stain signal in the dermal level of their wounds compared to uninfected, untreated (negative) controls (Figure 4b). There was, however, no significant difference between any treatment group and positive controls:

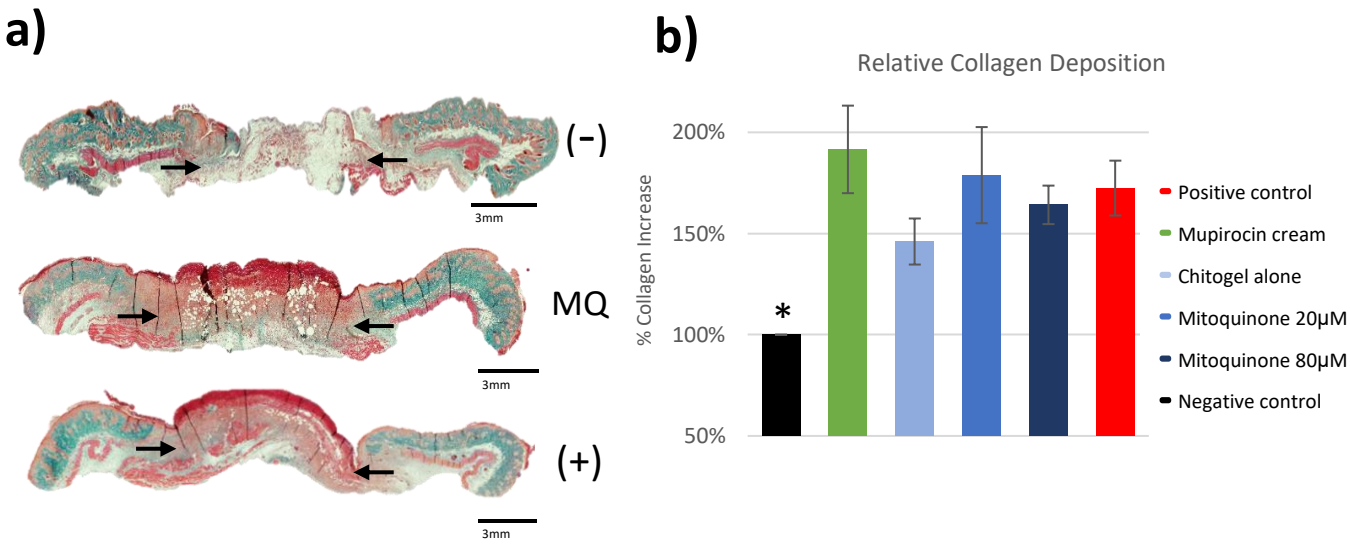


Figure 5a: Histologic images of dorsal wounds taken at end point with Masson's Trichrome staining, with arrows marking measured dermal layer of wound
(-) Negative control **MQ)** Chitogel with Mitoquinone 80μM **(+)** Positive control
Figure 5b: Percentage increase in green collagen signal in dermal layer of wound in Masson's Trichrome stain. Bars represent SEM. *=p<0.05 compared to positive controls

DISCUSSION

The present study reveals a novel finding that mitoquinone in Chitogel has potential as an antibiofilm treatment in infected wounds. This is in addition to its known wound healing benefits, which are shown here to be advantageous compared with conventional anti-staphylococcal treatments.

Chitogel with 80μM mitoquinone showed a significantly faster wound healing of *S. aureus* biofilm-infected wounds compared to untreated, infected controls. Both wound size and wound gape were significantly reduced by Chitogel with mitoquinone on macroscopic analysis. Interestingly, its wound healing effect was superior to both mupirocin and Chitogel alone, both of which did not differ significantly from infected controls. It is difficult to say if this was due to its antioxidant properties, its antimicrobial properties, or a combination of the two. The effect was confirmed on histologic analysis, where final wound size was only significantly reduced by Chitogel with 80μM mitoquinone. Faster wound healing gives mitoquinone an edge over existing antimicrobials like mupirocin, which has previously been linked to delays in wound contraction and an increase in pro-fibrotic factors such as wound fibroblasts, granulation tissue and extracellular matrix formation. [334]

Both the 20 μM and 80μM doses of mitoquinone in Chitogel reduced live bacteria in a biofilm to a similar degree to mupirocin. Chitogel alone did not have this effect, thereby indicating the potential of using mitochondrially-targeted antioxidants to augment existing wound treatments where there is a potential for infection or biofilm formation. A similar reduction was not seen in planktonic bacterial CFU counts from the wound tissue itself, or from wound bioluminescence, where no treatment was as effective as mupirocin.

One major difficulty in quantifying an anti-biofilm effect comes from the 3-dimensional nature of the biofilms. While mupirocin showed a clear superiority in reduction of bacterial bioluminescence over the gel treatment, this difference was not apparent in

other assays. Under these conditions, luminescence may have been more indicative of surface bacterial activity, where live bacteria were observed in greater concentrations on microscopy (Figure 3B). Bioluminescence also requires the presence of metabolically active bacteria, frequently being used as a measure of biofilm development and physiological activity of bacteria within biofilms. [335] Mupirocin may have caused the aforementioned discrepancy by reducing the metabolic activity of *S. aureus* biofilms more effectively than mitoquinone, as it is a bacteriostatic antibiotic that inhibits bacterial protein and RNA synthesis. [336]

Another difficulty in delineating the effectiveness of wound infection treatments is the immune response to *S. aureus* infection in mice. [337] In all temporal experiments during this study, the infection severity rose initially, before decreasing by the endpoint on day 9. A host immune response may behave as a confounder and result in differences between treatments and controls being more difficult to detect. These reflections highlight the importance of the multimodal approach to infection quantification adopted in this study, which allows these discrepancies to be detected and rationalised.

Collagen deposition, a by-product of fibroblast migration into the wound, did not differ to a significant degree between any of the treatments. The 9-day observation may have been too brief to adequately quantify any differences in collagen deposition during the proliferative phase of wound healing. The migration of fibroblasts into sterile murine wound models usually begins at day 3 and peaks after an average of 7 days, [338] at which time they begin producing extra cellular matrix, including collagen type I and III. [339] In humans, fibroblasts normally reach their maximum collagen production after 2 weeks, with overall wound collagen levels peaking within 3 weeks. [92] A more focused study with an appropriate length should be used to assess this effect using Masson's Trichrome staining. Interestingly, in the present study, these stains showed that infected mice had significantly higher levels of collagen signal than uninfected controls, echoing

previous findings that there may be a positive relationship between bacterial infection and fibrosis. [339]

Finally, mitoquinone has consistently shown that it is a safe treatment in both animal models and human trials. [246, 272, 274] The lack of adverse outcomes in the animal subjects or cytotoxicity against human cells reaffirms this observation. The data from the wound healing and wound infection assays supports further investigation of mitoquinone in larger animal models and, eventually, human clinical trials. Further directions may include optimizing its release from a vehicle medium at the tissue interface and testing its efficacy in mucosa and viscera, where an antimicrobial and wound remodelling benefit would be highly valuable for avoiding adhesions. [340, 341] This data will also aid in optimization of infected wound healing studies in small animal models, a significant body of work that continues to provide valuable insight into wound biofilm pathology and related bacteria–host interactions. [337]

CONCLUSION

Non-healing wound infections present a significant health and economic burden to patients and healthcare providers alike. Chitogel with mitoquinone is a promising antimicrobial and antibiofilm agent which, unlike traditional antimicrobials, may also convey a faster rate of healing and improved tissue remodelling.

Thesis Synopsis and Discussion

The studies included in this thesis have taught us much about the ‘delicate balance’ of reactive oxygen species within the sinuses, though much work remains to be done to fully harness this mechanism as a treatment for sinonasal adhesions, bacterial biofilms, and the healing process more generally.

The opening study of this thesis confirmed that ROS suppression from antioxidant or antibiotic exposure was associated with a slowed sinonasal cell migration across newly formed wounds *in vitro.* Perhaps the most interesting finding from this study was that this effect was observed more reliably and profoundly in sinonasal fibroblasts compared with epithelial cells. Such a disparity may create a situation whereby freshly formed surgical wounds in the sinuses have a reduced influx of fibroblasts, with a corresponding fall in collagen deposition, and are still able to epithelialize in a rapid fashion. In this way, ROS suppression may also suppress adhesion formation in the mucosa.

Another interesting finding in this first publication was the strength of this fibroblast migration reduction from the mitochondrially-targeted antioxidant mitoquinone. Originally included only as a ROS-suppressing control, its beneficial activity exceeded any of the other agents tested. This generated more interest in its role as an anti-adhesion agent, and lead to the unexpected discovery that this class of antioxidants was able to directly affect bacterial function by disrupting their membrane and osmoregulatory capability.

Through the studies that followed, we were able to demonstrate that mitochondrially-targeted antioxidants were active against staphylococcal biofilms and intracellular bacteria, and to establish a dose-response relationship for controlling sinonasal fibroblast migration through ROS suppression. Multiple toxicity assays confirmed a robust safety profile, provided that levels of ROS are not suppressed beyond the baseline level require for normal cell housekeeping functions.

These results were then unified and applied in an initial foray into *in vivo* experimentation, a murine infected cutaneous wound model, during which a degree of wound healing and anti-biofilm efficacy was demonstrated in tandem. There is much more to optimise here, but the groundwork has now been well and truly laid for further *in vivo* experimentation.

As expected, this work has generated several further research questions. Having demonstrated the safety and efficacy profile of an MTA in a murine cutaneous model of wound healing, it will now be interesting to see how this therapy performs in a larger sinonasal model. More focussed attention should also be given to the anti-adhesion efficacy of mitoquinone, using mucosal histology to assess fibroblast invasion, and the quantity and quality of collagen deposition after treatment. Drawing from our experience in the murine model, a longer observation period of 3-4 weeks in a non-infected mucosal wound would likely provide valuable data to confirm the *in vivo* efficacy of MTAs as beneficial wound remodelling agents. Provided that efficacy is proven here, human clinical trials measuring adhesion formation and biofilm persistence would then present a logical next step.

Modulation of reactive oxygen species represents a promising treatment avenue for chronic rhinosinusitis, in unison with existing treatments such as sinus surgery. Mitochondrially-targeted antioxidants have the potential to utilise this mechanism to address multiple factors driving disease persistence at once, providing hope for an illusive definitive treatment.

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