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ORIGINAL PAPER

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IN VITRO EVALUATION OF THE SINGLE AND MULTISPECIES BIOFILM PREVENTION CAPABILITIES OF TWO WOUND IRRIGATION SOLUTIONS AND A TOPICAL ANTISEPTIC

OCENA ZDOLNOŚCI DWÓCH ROZTWORÓW DO IRYGACJI RANY ORAZ JEDNEGO ANTYSEPTYKU DO HAMOWANIA BIOFILMU JEDNO- I DWUGATUNKOWEGO W WARUNKACH *IN VITRO*

ABSTRACT: **Aim** To investigate the biofilm prevention capabilities of 2 wound irrigation solutions and one topical antiseptic using single and mixed species *in vitro* assays. **Material and methods** A modified Calgary Biofilm Device (CBD) was used to determine the ability of 3 topical solutions (ActiMaris® (Quantum Medis), Prontosan® wound irrigation solution (B.Braun), and Octenisept® (Schulke)) to prevent the development of *Staphylococcus aureus* and *Pseudomonas aeruginosa* single species and mixed species biofilms. Evidence of biofilm development was assessed at 30 minutes, 3 hours, 24 hours, 48 hours and 72 hours. Biofilm material was recovered by sonication and quantified using colony counts. **Results** No *S. aureus* attachment was reported at 30 minutes or 3 hour when samples were treated with the test agents. The topical antiseptic Octenisept® prevented *S. aureus* biofilm growth for 72 hours, prevented *P. aeruginosa* biofilm growth up to 3 hours and continued to demonstrate multispecies biofilm inhibition at 72 hours. ActiMaris®, Prontosan® wound irrigation solution, did not prevent attachment of *P. aeruginosa* or mixed species biofilms. **Conclusion** If we accept the definition of a mature biofilm as one that has been forming for more than 10 hours, Octenisept® was the only solution that effectively prevented mature *S. aureus* biofilm development and mixed species biofilm development.

KEY WORDS: antimicrobial, biofilm, irrigation solution, wound

STRESZCZENIE: **Cel** Określenie zdolności dwóch roztworów do irygacji rany oraz jednego antyseptyku do hamowania biofilmu jedno- oraz dwugatunkowego w warunkach *in vitro*. **Materiał i metody** Do oceny zdolności dwóch roztworów do irygacji rany oraz jednego antyseptyku do hamowania rozwoju biofilmów jedno- i dwugatunkowych tworzonych przez *Staphylococcus aureus* oraz *Pseudomonas aeruginosa* zastosowano zmodyfikowany zestaw płytkowy typu Calgary (ang. Calgary Biofilm Device). Po 30 minutach, 3 godzinach, 24 godzinach, 48 godzinach oraz 72 godzinach od rozpoczęcia inkubacji płytek pokrytych badanymi preparatami z zawiesiną drobnoustrojów przeprowadzano test na obecność biofilmu. Masa biofilmowa odrywana była z urządzenia Calgary za pomocą sonikacji, a następnie poddawana posiewom ilościowym. **Wyniki** Każdy z badanych preparatów wykazał zdolność do zahamowania tworzenia biofilmu *S. aureus* na płytkach Calgary w czasie wynoszącym 30 minut oraz trzy godziny od rozpoczęcia inkubacji z zawiesiną drobnoustrojów. W przypadku zastosowania antyseptyku Octenisept® (Schulke), obecności biofilmu *S. aureus* nie wykryto także po 72 godzinach. Antyseptyk hamował również rozwój biofilmu *P. aeruginosa* po trzech godzinach od aplikacji i rozwój biofilmu mieszanego po 72 godzinach. Roztwory do irygacji rany ActiMaris® (Quantum Medis), oraz Prontosan® (B.Braun) nie hamowały tworzenia biofilmu *P. aeruginosa* oraz biofilmu mieszanego. **Wnioski** Octenisept® jest jedynym z badanych preparatów zdolnym do hamowania dojrzałego (starszego niż 10 godzin) biofilmu *S. aureus* oraz biofilmu mieszanego, współtworzonego przez *S. aureus* oraz *P. aeruginosa*.

SŁOWA KLUCZOWE: biofilm, rany, roztwory do irygacji, środki przeciwdrobnoustrojowe

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INTRODUCTION

The orderly and complex series of biochemical and cellular events that are involved in wound healing can, on occasions, be disrupted and require clinical intervention to restore order to the healing process [1]. A comprehensive approach to wound management (Wound Bed Preparation) focuses on optimising the condition of the wound bed so that an environment conducive to healing is achieved [2]. Advanced wound dressings and treatments will only work to their full potential when they are applied to a wound that has been suitably prepared prior to application [3].

Medicated wound irrigation solutions/gels are used to cleanse, moisten and decontaminate wounds either prior to treatment or between dressing changes. Saline is arguably the wound irrigation solution that is most commonly used as a topical wound cleansing agent. The physical process of irrigating a wound uses the pressure of the solution to assist in removing the debris and pathogens from the wound. It is considered that optimal wound irrigation is achieved the earlier it is commenced during the wound healing process [4]. More recently, wound irrigation solutions that are specifically designed to offer a targeted antimicrobial effect and thereby support wound healing have been made available [5]. Within this group surfactant based irrigation solutions have demonstrated a superior ability to target a mixed species infection compared to saline or antibiotic solutions [6]. Biofilms are recognised as bacterial communities that can actively impair wound healing [7]. Wound biofilms have demonstrated resistance to saline irrigation [8]. With this in mind this study investigates the ability of 2 wound irrigation solutions and a topical antiseptic to prevent the formation of single and mixed species *in vitro* biofilms.

MATERIAL AND METHODS

TEST SOLUTIONS

Solution 1 (ActiMaris®): 3% NaCl/0.2% NaOCl, hypochlorite based wound irrigation solution.

Solution 2 (Prontosan® wound irrigation solution): polyhexamethylenebiguanide (PHMB) (0.1%) with betaine.

Solution 3 (Octenisept®): octenidine dihydrochloride (0.1%) with 2-Phenoxyethanol (2.0%).

This work was funded by Schülke GmbH (Norderstedt, Germany), who also supplied all the test solutions.

TEST ORGANISMS

A biofilm-forming strain of *S. aureus* (ATCC 12600) and *P. aeruginosa* NCIMB 10434 were investigated [9]. These strains were chosen for their proven biofilm forming ability *S. aureus* and *P. aeruginosa* are recognised as common colonising wound pathogens.

INOCULUM PREPARATION

STAPHYLOCOCCUS AUREUS

Overnight cultures of *S. aureus* were adjusted to an OD of 0.217 (570 nm) using Tryptic soy broth (TSB). This suspension was then diluted using TSB in order to give an initial inoculum concentration of 3.75×10^5 cfu/ml. In each instance the initial inoculum concentration validation was validated using plate counts.

PSEUDOMONAS AERUGINOSA

Three colonies of *P. aeruginosa* grown on Tryptic soy agar were inoculated into 5 ml TSB. The inoculum was diluted using TSB in order to produce an initial inoculum concentration of 2.5×10^5 cfu/ml. In each instance the initial inoculum concentration validation was validated using plate counts.

MULTI-SPECIES INOCULUM

In order to produce multi-species inocula the single species suspensions described were mixed in equal quantities.

PREVENTION OF BIOFILM FORMATION

The modified Calgary biofilm device CBD experiment involved a 2 component vessel that consisted of a 96 well plate and a polystyrene lid with 96 pins (transferable solid phase screening system, Nunc, Sourced from Fisher Scientific UK Ltd.). The system fitted together so that each pin was suspended in an independent well.

Microtitre plate wells were filled with 150 µl of the test solution. A different test solution occupied each row. Column 1 contained the commercial concentration of the solution and subsequent columns contained 1:1 TSB dilutions of the previous well. The doubling dilutions were carried out within the microtitre plate. Column 12 contained TSB only (positive control). A pin lid was placed onto the microtitre plate and the device was left to stand for 1 minute to encourage adherence of the test solutions to the test pins. After 1 minute the lid was transferred into an empty microtitre plate and allowed to dry in a fume cupboard for 15 minutes.

A challenge plate containing 150 µl of inocula per well was prepared. After drying the pin lid was placed onto the challenge plate, sealed and incubated at 37°C on an orbital shaker (50 rpm). Plates were incubated for: 30 minutes, 3 hours, 24 hours, 48 hours or 72 hours.

Following incubation the pin lid was transferred to a new microtitre plate containing 150 µl sterile PBS per well. The plate and lid were agitated gently for 30 seconds to remove planktonic bacteria from the pins. The washing stage was repeated 3 times in fresh PBS the lid was then placed into a fresh plate containing 150 µl sterile TSB. Attached bacteria were recovered in the TSB by placing the recovery plate into a sonic water bath for 15 minutes. The number of bacteria in the recovery medium was quantified using 10 µl drop plates.

Inhibition of biofilm growth was recorded when no bacteria were recovered from the biofilm surfaces. Then treatment resulted in a reduction in recovered biofilm compared to the control biofilm inhibition was recorded as recovery below 1×10^4 cfu/ml as this represented a 3 log reduction from the positive control samples.

All experiments were carried out in triplicate.

RESULTS

SINGLE SPECIES BIOFILM PREVENTION

STAPHYLOCOCCUS AUREUS

At the commercial concentration all the wound care solutions prevented biofilm formation at 30 minutes and 3 hours. From 24 hours onwards only Octenisept® prevented bacterial attachment. No bacteria were recovered from the Octenisept® treated samples at any of the time points when it was used at the commercial concentration (Table 1). Dilution of Octenisept® resulted in bacterial growth equivalent to the control samples at 24 hours, 48 hours and 72 hours.

The biocidal activity of all the agents decreased with time. At 30 minutes all the solutions prevented *S. aureus* biofilm development even after considerable dilutions (Table 2). By 3 hours the concentration required to inhibit biofilm growth was greatly increased for all the agents.

PSEUDOMONAS AERUGINOSA

Octenisept® was the only agent that effectively prevented the formation of *P. aeruginosa* biofilms (Table 2). Octenisept® was active up to 3 hours but did not inhibit *P. aeruginosa* biofilm growth after 24 hours. Octenisept® was only active when tested at the commercial concentration.

MIXED SPECIES ASSAY

Octenisept® successfully prevented the formation of mixed species biofilms for up to 72 hours (Table 3). The agent was active after 1 dilution at 30 minutes and 3 hours however by 24 hours the agent only prevented biofilm formation at the commercial concentration. At the commercial concentration Prontosan® wound irrigation solution inhibited mixed species biofilm growth so that 6.7×10^4 cfu/ml biofilm bacteria were recovered from the samples at 30 minutes. The other agents did not successfully prevent or significantly inhibit the formation of mixed species biofilms.

Table 1.Time points at which the solutions resulted in complete inhibition of *S. aureus* biofilms.

Solution	30 minutes	3 hours	24 hours	48 hours	72 hours
1	0	0	1	1	1
2	0	0	1	1	1
3	0	0	0	0	0
Control	1	1	1	1	1

0 – biofilm inhibition, 1 – biofilm growth.

Table 2. Average concentration of the agent required to prevent biofilm formation. Prevention of biofilm formation was defined as a bacterial recovery below 1×10^4 cfu/ml. Control samples resulted in a recovery of 1×10^7 cfu/ml.

Agent	30 minutes	3 hours
1	6.3%	25%
2	0.1%	100%
3	0.1%	50%

Table 3. Time points at which the solutions inhibited growth of mixed species biofilms.

Solution	30 minutes	3 hours	24 hours	48 hours	72 hours
1	1	1	1	1	1
2	1	1	1	1	1
3	0	0	0	0	0
Saline	1	1	1	1	1

0 – biofilm inhibition, 1 – biofilm growth.

DISCUSSION

P. aeruginosa biofilms have been regarded as mature once after 10 hours of growth [10]. Extrapolating this definition to other species, Octenisept® effectively prevented *S. aureus* mature biofilm growth. Octenisept® effectively prevented 24 hour *P. aeruginosa* biofilm formation but did not continue to prevent the formation of biofilms when the surface was exposed to an inoculum for longer than 24 hours. This suggests that *in vivo* a single treatment with Octenisept® would not be capable of inhibiting *P. aeruginosa* biofilm formation for 72 hours. However if it was used daily prior to development of a mature biofilm, it may help to prevent the development of a mature biofilms that could ultimately retard the healing process. Octenisept® prevented the formation of a mixed species biofilm over the 72 hour test period. The ability to inhibit mixed species biofilm formation may primarily be linked to the ability of Octenisept® to prevent *P. aeruginosa* biofilm formation. *In vivo* biofilms can be assumed to be multi species, comprising of more than 2 species, thus making comparisons with this *in vitro* situation less straight forward. In addition, the complexity of organic molecules within a wound together with inconsistencies in adsorption of the test agents confounds direct comparison.

Surfactant containing irrigation solutions have previously demonstrated an improved antimicrobial activity compared to saline and antibiotics [6]. Octenisept® contains the amphoteric surfactant cocamidopropylbetaine that acts to reduce the surface tension of aqueous solutions [11]. Along with antibacterial agents such as octenidine dihydrochloride, these agents are thought to contribute to the biofilm prevention capabilities of Octenisept® wound irrigation solution.

The primary aim of an irrigation solution is to remove debris from the wound, however this data demonstrates that appropriately chosen wound irrigation solutions may also provide an antimicrobial and furthermore an anti-biofilm effect. Appropriate use of a wound irrigation solution should therefore be considered as part of the Wound Bed Preparation protocol.

Wound irrigation solutions are typically used on multiple occasions throughout the wound healing process. In this study the solutions were not replenished and therefore further applications of the solutions are likely to enhance the anti-biofilm effect. This data suggests that the growth of potentially pathogenic organisms such as *S. aureus* can be inhibited for up to 72 hours. The lavaseptics investigated, ActiMaris® and Prontosan®, were unable to completely stop the *P. aeruginosa* and mixed species biofilm development. The highest capability of such biofilm eradication was displayed by the Octenisept® antiseptic. It is more difficult to control the growth of other pathogens such as *P. aeruginosa* and mixed species cultures. *P. aeruginosa* and mixed species cultures may benefit from repeated application of the agent at 24 hour intervals and from concurrent treatment with a targeted antimicrobial in case of lavaseptics. In the era of increasing antibiotic resistance of microorganisms, combination of antiseptic with lavaseptics containing antimicrobial substance and displaying pharmaceutical compatibility are potentially a way to obtain an acceptable outcome and to avoid side effects related to the cytotoxicity and spread of drug-resistant microbes.

Wound irrigation solutions can be used on acute or chronic wounds. This data suggests that the use of appropriate wound irrigation on an acute/surgical wound may help to prevent biofilm formation in these circumstances by providing infection prophylaxis. This intervention would be most appropriately employed in those patients considered to be at a higher risk of infection than the "average" patient. This study does not address the role of wound irrigation solutions on the removal of biofilms and thus the implications of this data on chronic biofilm infected wounds requires further investigation.

This work utilises *in vitro* assays to test the activity of antimicrobial agents. The situation is much more complex within a chronic wound. The material available for surface attachment are more irregular and biological in nature. In addition, agents are commonly diluted by wound fluids. This study attempts to address agent dilution effect by testing a range of concentrations. In addition, wound fluids contain proteins, immunological cells, growth factors, cytokines and proteases, some of which support healing whilst others inhibit the proliferation and migration of new fibroblast cells. These biological cells may influence the activity of the agent and also the ability of the wound to heal. This level of complexity is not addressed in this study and should be considered when interpreting the *in vivo* implications.

Generally in this study it was found that the agents either prevented biofilm growth completely, or results were in line with the control samples. This is not surprising since regulatory claims and requirements related to minimal toxicity, as well as cost constraints, encourage the use of minimal concentration of active ingredients. This study supports previous studies that demonstrate that when aiming to treat biofilm forming bacteria, as opposed to planktonic isolates, minimum active concentrations can rapidly become ineffective.

CONCLUSIONS

Wound irrigation solutions may help to prevent the formation of biofilms within wounds. Knowledge regarding the antimicrobial and anti-biofilm potential of different wound solutions should be used to inform irrigation solution choice as a component of Wound Bed Preparation, in order to support an effective healing process.

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