

## **Efficacy of Various Wound Irrigants against Biofilms**

### Introduction

The formulation that only a clean wound can undergo problemfree secondary healing highlights one insight into modern wound management and constitutes a basic tenet of surgical wound treatment [1,7,10]. To that effect, the wound should be thoroughly and radically cleansed to remove necrotic tissue, but this should be carried out gently to preserve the granulation tissue.

In addition to factors relating to the underlying disease and to macro- and microangiopathic disorders, every wound is influenced by wound deposits containing debris as well as microbial loads. As such, chronic wounds in particular are colonised by microorganisms. While these microbial colonies composed primarily of bacteria constitute a complex microbiological ecosystem, they have hitherto only rarely been analysed. As the chronification process unfolds, and regardless of the primary pathophysiological cause (of injury), typical bacterial species tend to be selected, thus providing for “uniformity” of the microbial spectrum of chronic wounds [12,21,22].

This insight alone would suggest that a more intensive scrutiny of the wound as a complex “ecosystem” is absolutely imperative. The hitherto practice of analysing individual microbial isolates and of reducing/eliminating them locally is being increasingly viewed as of little benefit [4].

In particular, the classic approach did not take adequate account of the actual microbial biotope character of a wound with the potential to form a biofilm. But a native biofilm embodies a special symbiotic form of life with different species of bacteria living together, possibly with fungi. It develops initially from isolated free-swimming (planktonic) bacteria. These form colonies on the most diverse natural surfaces (teeth, gallstones, skin, wounds, etc) and on synthetic surfaces (endoscopes, water pipes, fermentation tanks, etc) where they multiply. A special characteristic of all biofilms is their differentiated structure analogous to that of higher forms of tissue. Accordingly, the term “biofilm” has been defined as “bacterial cells that attach to a substrate, (surface/boundary surface) and are fully embedded in a polymer organic mass (matrix) produced by the bacteria themselves”. This matrix is composed of highly aqueous biopolymers (polysaccharides, proteins, glycoproteins, alginates) as well as of other aqueous compounds. A biofilm can harbour a most diverse range of bacterial species with a density of up to  $10^{11}$ /ml [25].

In evolutionary terms, the organisational structure of a “biofilm” confers important localization and selective advantages on the bacteria. Whereas individual bacterial cells in a planktonic form of growth are directly exposed to attack from the outside environment, the biofilm offers protection against the effects generated by cleaning solutions, antiseptic or antibacterial substances as well as against the cellular defence mechanisms of the macroorganism. A comparison of both organisational forms using an identical bacterial species revealed that the biofilm was endowed with a 1,000-fold greater minimal inhibition concentration than planktonic suspensions [11].

This extraordinarily high resistance evidenced by bacterial biofilms to antibacterial substances provides an explanation for a number of chronic infections for which only inadequate treatment is available, e.g. caries, cystic fibrosis, bacterial endocarditis, bacterial prostatitis, otitis media, osteomyelitis.

To date, various semi-quantitative methods have been employed for direct detection of biofilms. However, these did not provide for exact quantification of the biofilm. Likewise, audioradiography, epifluorescence microscopy as well as a combination of both methods have been used for detection purposes. However, these were able to attest to the presence of viable but not of dead biofilm microorganisms [2,6,17,18]. Kinniment and Wimpenny [14] reported on the production of biofilms in a special fermenter which were then removed with sharp blades and investigated for enumeration of viable microorganisms using staining procedures and microscopy. It was not until 1999 that a multi-step culture and quantification method, specially tailored to biofilms and based on endotoxin analysis, was first patented [8].

To date, only marginal attention has been paid to the phenomenon of the biofilm as regards (cleansing) management of chronic wounds [13,19,20]. Already back in 1988 Brook [5] showed how microorganisms mutually support each other when it comes to pathogenicity. Trengroce and Stacey produced data in 1996 [26] to demonstrate that microbial synergistic activities triggered wound healing disorders and in 2003 Stewart [24] proposed an effective principle of action for elimination of biofilms. Very recently, Ziegler et al [27] stated that biofilm-mediated loads had first of all to be removed mechanically from wound surfaces.

To date, mainly a classic isotonic saline solution as well as Ringer's solution, and over the past few years antiseptic solutions as medicinal products, have been employed to clean chronic wounds. However, it remained to be elucidated whether, and to what quantifiable extent, biofilm is eliminated by these solutions. In the study presented here we therefore investigated the efficacy of these solutions on the basis of a biofilm of *Pseudomonas aeruginosa* grown on silicone surfaces.

## **Materials and Method**

### **Materials**

- Isotonic saline solution (Isotonic NaCl), Fresenius Kabi, Bad Homburg
- Ringer's solution (RL), B.Braun, Melsungen
- Prontosan W, Prontomed, Hiddenhausen (0.1 % solution of poly(imino[imidocarbonyl]imino[imidocarbonyl]-iminohexamethylene) hydrochloride (trivial name Polyhexanide, PHMB)).
- Distilled water; Ampuwa<sup>®</sup>, sterile, pyrogenfree water, FMC, Bad Homburg
- Silicone surfaces (used to make control and test carriers); 8 mm internal Ø, 12 mm external Ø; Deutsch-Neumann, Berlin
- Laboratory equipment to measure unbound chlorine and pH value
- Microtiter-plate reader "Sunrise", Tecan, Crailsheim as well as endotoxin lysates and standards from the firm Charles River, Sulzfeld/Germany

### **Method**

#### **Microbial identification and culture**

4 wild types of the species *Pseudomonas aeruginosa* were identified in the laboratory and cultured from single colonies. Following growth in trypticase soybean broth (TSB), the bacteria were washed 3x by means of centrifugation at 3000 rpm and

resuspension in Ringer's solution and then set in water of standardised hardness (WSH [3] to a concentration of  $10^6$  colony forming units (cfu/ml).

### **Biofilm growth**

A cleaned silicone tube was incubated with the aforementioned microbial suspension over a 10-week period at a temperature of  $36 \pm 1$  °C, while using permanently circulating pumps. Following the culture phase, the silicone tube bearing the biofilm was cut into sections for use as test and control carriers (with test surfaces measuring  $17.6 \text{ cm}^2$  in each case) and deep-frozen at  $-20^\circ\text{C}$  until the time of use.

### **Calculation of baseline load using control carriers**

The microbial count of the grown biofilm was calculated using control carriers (n=16) as per the following method for release and investigation of the endotoxin content [IU/ml] (calculation of the biofilm equivalent). From the endotoxin content of the control carriers the arithmetic mean value (representing the baseline load), standard deviation as well as the variation coefficient were calculated.

### **Incubation of test carriers in the irrigants**

At least 8 test carriers harbouring biofilm were incubated in 350 ml of the irrigants, listed above, at a temperature of  $36 \pm 1$  °C using an exposure time of 24 hours.

### **Calculation of the biofilm equivalent**

On expiry of the exposure time, the test carriers were rinsed 3x 3 sec using a flow velocity of 3 m/s and then, just as were the control carriers, investigated for residual biofilm load.

To that effect, the test and control carriers were incubated in a disintegration solution as per the Biofilmyl® method [8] for an exposure time of 30 min at  $25^\circ\text{C}$  to release the endotoxin from the Gram-negative bacteria harboured by the biofilm.

Then the biofilm eluate was emptied into an endotoxin-free test tube and the endotoxin content was calculated using the kinetic turbidimetric method.

Proof of complete biofilm detachment was obtained for each set of silicone tubes by repeatedly subjecting the control carriers to an elution process, thus checking for, and ruling out, residual endotoxin concentrations.

To compare and evaluate the efficacy profiles of the irrigants against biofilm, mean values and standard deviations were calculated for the endotoxin content measured for the respective test carrier group. Reduction factors vis-à-vis the baseline load served as evaluation parameters.

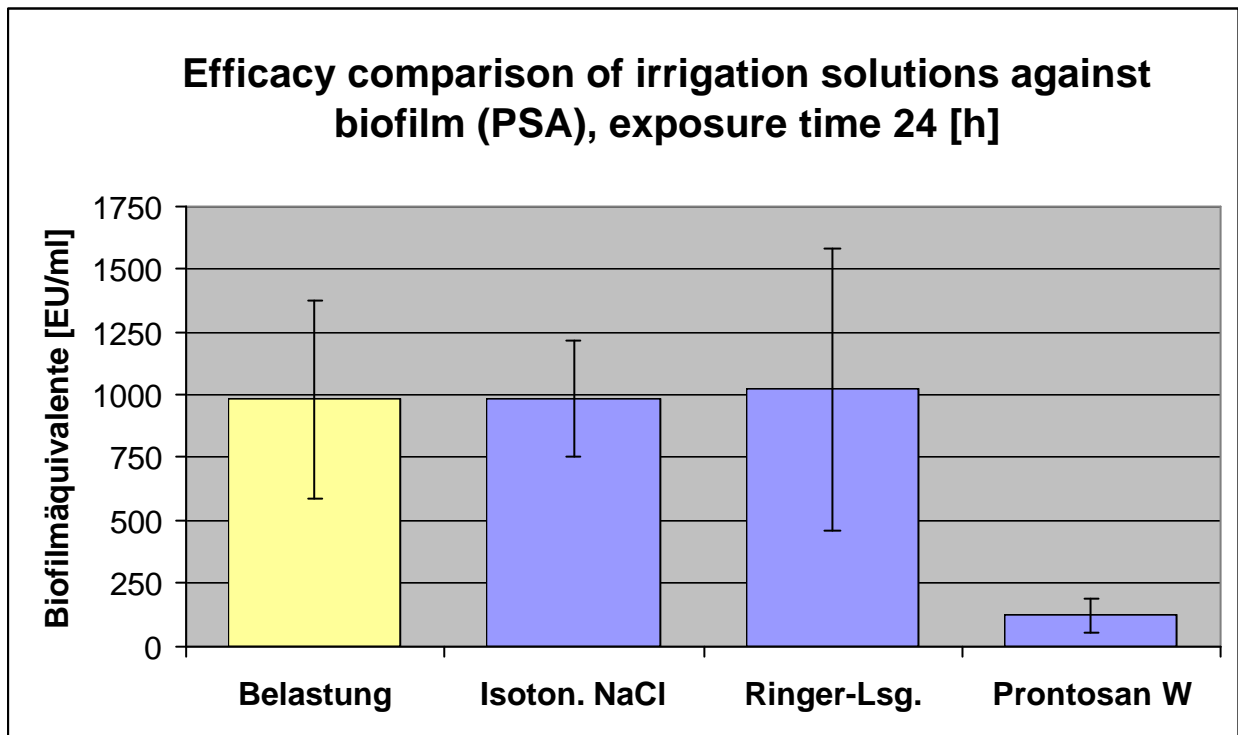
## **Results**

### **Baseline load on the control carriers**

Having examined the control carriers, an arithmetic mean value of 983 EU/ml was calculated as baseline load (biofilm equivalent). The standard deviation was 394 EU/ml and the variation coefficient 40 %.

## Biofilm equivalent after exposure to the irrigants

The efficacy test results obtained for the irrigants did not produce any evidence that the baseline biofilm load had been affected by the isotonic solution ( $984 \pm 233$  EU/ml) or by Ringer's solution ( $1022 \pm 557$  EU/ml). Conversely, Prontosan® W had reduced the biofilm baseline load after 24 hours by a factor of 7.8 ( $125 \pm 67$  EU/ml  $p < 0.001$ ).



Load    Isotonic NaCl    Ringer's solution    Prontosan W

Biofilm equivalent [EU/ml]

### Figure 1

Endotoxin content (biofilm equivalent) for species *Pseudomonas aeruginosa* (PSA) on control carriers (before exposure) and after 24-hour exposure to various irrigants

### Discussion

The Biofilmy® method [8] employed here is a multi-stage culture-identification method. First of all, individual species of bacteria were grown under aseptic conditions in an aquatic system to give a homogeneous biofilm; in the present study *Pseudomonas aeruginosa* was grown on silicone surfaces. Then the biofilm, including the homogeneously distributed bacterial cells contained therein, was completely detached, disintegrated and the endotoxins contained in the bacterial cell walls released. The endotoxin content was then quantified using the Limulus amoebocyte lysate (LAL) test which has been available for years as a commercial kit

in the pharmaceutical industry and has become the gold standard throughout Europe in pyrogen diagnostics [9]. On the one hand, a control carrier group was formed from the silicone surfaces harbouring growth of *Pseudomonas aeruginosa* biofilm; from this was calculated the endotoxin baseline load [EU/ml] as a biofilm equivalent. With a variation coefficient of 40%, this group showed little scatter for a biological system, instead showing homogenous distribution of the bacterial cells or of the biofilm.

In addition, a test carrier group was formed from the silicone surfaces harbouring biofilm growth so as to investigate the efficacy of wound irrigants. By adopting this approach for our study, we used a method that differed radically from the methods employed hitherto, which were mainly based on elucidation of the microbial count of viable organisms that happened to be eliminated from biofilms but were not representative of the latter's potential microbial complement. However, it was not possible to detect those microorganisms lodged within the biofilm (viable or dead). Hence on using these classic methods it was not possible to establish a correlation between the microorganisms eliminated from wounds using irrigants and the actual biofilm load on the (wound) surfaces.

Conversely, the Biofilmyl<sup>®</sup> method employed in our study is the first of its kind to provide for exact quantification of the biofilm load on a surface before and after exposure to irrigants. In this respect it must be pointed out that it is possible that different efficacy results would be obtained were one to use other species rather than the *Pseudomonas aeruginosa* species used here. However, in view of the ubiquitous nature of this bacterium coupled to its pivotal role as a human pathogen these initial findings would appear to be justified.

The special organisational structure of a biofilm largely accounts for the persistence of bacterial infections in fistulas and wounds in the human body and, regardless of the disease entity, is scarcely amenable to elimination by mechanical or antibiotic interventions [23]. It is therefore of paramount importance in wound management to know what is the potential of irrigants to eliminate biofilms.

In our study in addition to the classic isotonic saline solution as well as Ringer's solution, we deliberately chose an irrigant based on polyhexanide because this substance is endowed with the broadest therapeutic spectrum [16] and data are available to corroborate its very low level of inhibition of the granulation potential compared with the substances povidone iodine and, in particular, octenidine [26].

The test results demonstrated that after 24-hour exposure neither the isotonic saline solution nor Ringer's solution had any impact on the *Pseudomonas* biofilm. Conversely, the performance of the surfactant irrigant (0.1% undecylene acid amidopropyl betaine) in combination with 0.1% polyhexanide proved to be highly significant against the biofilm baseline load. This effected an 87% reduction in the biofilm growth originally encountered.

These initial findings lend credence to the belief that apart from irrigants, antiseptic solutions should also be investigated for their efficacy at eliminating biofilm. The efficacy of irrigant and antiseptic solutions against biofilms can then be evaluated in respect of the other parameters that are of relevance to wound healing: "effect on granulation potential" as well as the "therapeutic spectrum".

As far as the clinical practice of biofilm removal based on moist management practices is concerned, our investigations attest to the superior efficacy of the surfactant and polyhexanide solution compared with isotonic saline or Ringer's solution. It thus seems advisable to investigate in large clinical trials this superior effect demonstrated in the laboratory.

[Abstract schon vorhanden]

## References