The Biofilm-forming capacity of *Staphylococcus aureus* from chronic wounds can be useful for determining Wound-Bed Preparation methods

**ABSTRACT**

**Introduction:** *Staphylococcus aureus* is frequently found in chronic wounds. Bacterial biofilms within chronic wounds impact on the surgical closure of the wounds with skin grafting. Methods of wound bed preparation (WBP) influence the microorganisms within the biofilm. We used *in vitro* monitoring to analyse the possible influence of WBP methods on the *in vivo* formation of biofilms.

**Aim:** To conduct a comparative analysis of the impact of WBP treatment methods on the capacity of *S. aureus* isolated from chronic wounds to form biofilms *in vitro.*

**Methods:** We modeled *S. aureus* biofilm formation in 96-well plates. We assayed the capacity of bacteria isolated from chronic wounds to form biofilms at the time of patient admission and after treatment with either ultrasound debridement (UD) and topical negative pressure (TNP; Main Group) or standard dressings (Control Group). We also compared biofilm formation by bacteria from patients with different grafting outcomes.

**Results:** The treatment of chronic wounds with UD and TNP reduced the capacity of *S. aureus* to synthesise a major biofilm substance. *S. aureus* isolates from patients with favourable skin-grafting results had a lower capacity to form biofilms *in vitro* compared with isolates from patients with poor skin-grafting results. The use of UD and TNP for surgical closure reduced the length of the skin-graft healing process compared with the use of standard bandages.

**Conclusion:** The isolation from chronic wounds of significant titers of *S. aureus* strains with a high capacity to form biofilms within 2 to 6 hours of incubation *in vitro* highlights the advantage of using hardware methods (UD and TNP) for WBP for surgical closure.

**INTRODUCTION**

All chronic wounds harbor a diverse microflora that contributes directly and indirectly to their non-healing phenotype[1,2]. Exposed and devitalised tissue in chronic wounds provides a favourable environment for colonisation by a wide variety of microorganisms[3,4]. Frequently, chronic wounds harbor aerobic gram-positive cocci (*Staphylococcus aureus* is reported to be present in frequencies varying from 43% to 88% of the ulcers)[5,6]. Biofilms are a significant factor that differentiates chronic wound infections from acute wound infections[3]. The key characteristics of biofilm-forming bacteria are their resistance to host defenses and their tolerance of antimicrobials[7,8]. Bacterial biofilms are a complex microenvironment consisting of single or mixed species that are attached to one another or to surfaces and encased within extracellular polymeric substances (EPSs)[9]. The EPS generally consists of polysaccharides, and the bacteria within the EPS induce chronic inflammation that delays healing[10,11]. The polysaccharide components of *S. aureus* cell walls facilitate adherence to extracellular matrix components such as fibronectin or collagen in wounded tissues[1,2]. Different methods of treating pathogenic biofilms in chronic wounds can improve patient outcomes[3,7,11].

Sibbald *et al.* (2000) and Falanga (2000) first described the concept of wound bed preparation (WBP)[12,13]. The approach stresses that the successful diagnosis and treatment of chronic wounds require holistic care and a team approach[14,15]. The three main objectives of care in the WBP model are debridement, bacterial balance, and exudate management. The achievement of the key objectives should lead to a well-stabilised wound supported by a sufficiently vascularised wound bed.
Debridement promotes healing by removing non-viable tissue and biofilms that shield bacterial colonies\(^\text{[16]}\). Ultrasonic assisted debridement (UD) is a relatively painless method of removing non-viable tissue. UD allows a rapid transition to secondary procedures, and its effectiveness in biofilm disruption is supported by in vitro studies and a decreased bioburden in patients\(^{[17,18]}\). Topical negative pressure (TNP) is reported by clinical and experimental studies to decrease bacterial colonisation. The seal created by the foam and drape used to apply TNP reduces the risk of bacterial colonisation; and TNP causes improved blood perfusion that may increase resistance to infections\(^{[19]}\). The complex use of UD and TNP methods in WBP prior to skin transplant may impact on biofilm formation following the transplant and thus reduce the frequency of post-surgery complications.

Our study aimed to conduct a comparative analysis of the in vitro biofilm-forming capacity of \textit{S. aureus} isolated from chronic wounds before and after wound treatment with various WBP treatment methods (standard bandages or UD/TNP) prior to skin transplant.

**MATERIALS**

We enrolled 55 patients (20 females and 35 males, aged 25 to 70 years) who sought treatment for chronic wounds at the Gomel Regional Centre for Thermal Injury, Wound, Wounds Infection, and Reconstructive Surgery (Gomel City Clinical Hospital No1, Belarus) during 2011. The chronic wounds consisted of venous leg ulcers (n=16), pressure ulcers (n=6), traumatic ulcers (n=10), and inflammatory ulcers (n=13). In each case, the duration of the ulcers was more than 4 weeks prior to the patient’s enrollment in the study. Because UD and TNP are already used in the Gomel Regional Centre to treat chronic wounds, approval from the local ethical committee was not necessary. All patients gave written informed consent before being admitted to the study. In all cases, prior treatments, including domiciliary outpatient treatment with local remedial therapy and empirical antibiotic therapy, were unsuccessful. At the time of admission to the Gomel Regional Centre, each patient had wounds with clinical signs of inflammation.

**METHODS**

We divided the patients into two groups, depending on the treatment methods. The pre-procedural wound-treatment protocol for the Control Group (n=30) included antiseptic bandages, chlorhexidine, povidone iodine, and polyethylene glycol-based ointments (e.g., Levomecol). We performed WBP 7-10 days after starting the pre-procedural treatment. We changed the patients’ bandages once each day. After the pre-procedural treatment and WBP, the patients received skin grafts.

The pre-procedural wound-treatment protocol for the Test Group (n=25) included UD and TNP. We applied UD at 25 kH using a «Sonoca-185» (Soring Inc., Germany) apparatus. Each patient underwent two UD treatments. We conducted the first UD treatment on the 2nd or 3rd day after beginning the pre-procedural wound-treatment protocol. We performed TNP each day for 5 to 7 days using the Visma-Planar (Belarus) negative pressure-therapy system. The optimal target pressure was 75-125 mmHg; the pressure was dependent on the patient’s tolerance. We changed the wound dressings for the patients in the Test Group every 48 hours. After receiving TNP, the patients in the Test Group underwent a second UD procedure and concomitant skin grafting.

We performed wound closure for the patients in both groups using a split-thickness (0.4 mm) skin graft. None of the patients had intra-procedural complications. We changed the wound dressings for the patients in both groups once per day during the post-procedural period.

**OUTCOME ASSESSMENT**

We collected data characterising the clinical efficacies of the pre-procedural treatments via detailed visual assessment of the regeneration process. Our data included the amount of wound effluent, the condition of the tissue surrounding the wound, the degree of granulation, the wound size and surface condition, and the appearance of edge epithelialisation.

To assess the effectiveness of the skin grafts, we considered the recommended clinical signs of wound readiness: absence of inflammation, expressed exudation, purulent discharge, wound adhesiveness, mature red or bright pink granulations, and the presence of edged epithelialisation\(^{[12]}\). We assessed the process of graft healing by considering not only the terms and signs of the fixation, but also the complete graft healing, including the colour and degree of fixation of the graft and the degree of exudation after surgical wound closure. We defined the grafting procedure as successful when graft fixation occurred on the 3rd or 4th day after surgery and complete graft healing occurred within 7 to 9 days after surgery. We determined the presence of graft instability based on paleness and graft failure. We defined graft failure as rejection or dissolution of the graft in the immediate post-procedural period (day 8, ±3 days, on average). If a graft failed, then the patient required future repetition of the procedure.
BACTERIAL STRAINS

We isolated *S. aureus* from chronic wounds. Previous work by our group demonstrated that *Staphylococcus* species predominate in cultures isolated from chronic wounds [20].

BIOFILM ASSAY

We modeled biofilm formation in sterile 96-well polystyrene microtiter plates [21]. We used Congo red and crystal violet stains to visualize both the matrix and the bacterial cells [22, 23]. We used 95% ethanol to extract the stain connected to the biofilm. We measured the optical density (OD) of each sample at a wavelength of 540 nm (for crystal violet/ethanol solution) and 490 nm (for Congo red/ethanol solution) using a Sirio microplate reader (Seac Radium Group, Italy).

We isolated *S. aureus* from the patients’ wounds before and after WBP and compared the biofilm formation capacities of the isolates from patients with different skin-grafting outcomes. We performed all of our experiments in the Clinical-Diagnostic Laboratory of the Republican Centre of Radiation Medicine and Human Ecology and at the Clinical Laboratory Diagnostic Department of Gomel State Medical University (Gomel, Belarus). We presented the results as medians with lower and upper quartiles (M [25–75%]). We used a non-parametric Mann-Whitney U-test to compare the two study groups the Wilcoxon test to compare dependent samples (variables). We considered results to be statistically significant when *p*<0.05.

RESULTS

By 7 to 10 days after the pre-procedural treatment, all of the patients in the Control Group exhibited decreased clinical signs of inflammation such as erythema, edema of peri-wound tissues, exudate volume, and release-from-wound detritus. In addition, the growth of friable granulation tissue began after the patients received the standard-dressing treatment. After we performed the standard-dressing treatment, the wounds of all of the Control Group patients were ready for plastic closure, and the patients received skin grafts.

After the first application of UD to the patients in the Test Group, all of the necrotic areas were successfully debrided. The application of TNP resulted in the purification of the wound, the activation of reparative processes, and the covering of the bottom of the wound with granulation tissue. As a result, the patients in the Test Group experienced more noticeable clinical improvement following the skin grafting (7th, 10th day of treatment) than the patients in the Control Group. The patients in the Test Group exhibited no signs of inflammation and no wound discharges. In addition, we observed signs of active reparation in the patients in the Test Group immediately following the grafting procedure.

Differences in the graft healing dynamics of the patients depended on the pre-procedural treatment. All of the patients in the Test Group experienced successful wound closure: skin graft fixation occurred by the 3rd day with complete healing by the 9th (6th; 10th) day. The results were different for the patients of the Control Group. In the Control Group, 80% of the patients (n=24) had complete graft healing. The patients in the Control Group, however, exhibited several signs of instability during the post-procedural treatment; such as weak fixation, paleness, and exudation from under the grafts within 4 to 5 days; resulting in additional interventions. As a result, complete graft healing occurred significantly later in the Control Group than in the Test Group (12th [10th; 15th] day [*p*=0.015]) (Fig. 1). Twenty percent of the grafts in the Control Group did not take during days 2 to 5 of the post-procedural period. Some of the patients in the Control Group developed graft loss that required supplementary conservative treatment and repeated skin-grafting procedures.

The patients in both groups received a dynamic bacteriological examination. In total, we detected 45 strains of *S. aureus* at a diagnostically significant titer (>10^5 c.f.u.). We isolated 20 strains from patients in the Control Group and 15 strains from patients in the Test Group. We considered bacterial contamination to be significant at a titer

![Fig. 1. Complete graft healing terms of the patients with different WBP treatment methods.](image-url)
of $10^3$-$10^5$ c.f.u. At the first bacteriological examination, all of the isolates had titers >$10^5$ c.f.u. After treatment with UD and TNP, the titers decreased up to $10^2$-$10^5$ c.f.u. In all of the patients in the Control Group, the titers remained high, more than $10^5$ c.f.u., prior to surgery. During the post-procedural period, we identified 14 strains of *S. aureus* with titers of $10^3$-$10^5$ c.f.u. in the patients in the Control Group. During the same period, we identified nine such strains in the patients in the Test Group.

Figure 2 shows the *in vitro* biofilm-forming capacity of the *S. aureus* strains isolated during the first bacteriological examination (Fig. 2). We noted an increase in the OD of the crystal violet-stained eluate from 0.171 (0.057; 0.308) units at 2 hours to 0.568 (0.281; 0.611) units by 4 hours (*p*=0.015). This change reflects the active reproduction of *S. aureus*. During the remaining incubation time (from 4 to 48 hours), the biomass of the *S. aureus* isolates in the model biofilms was stable. An increase in the OD of Congo red-stained eluate (*p*=0.03) indicated a change in the dynamics of the synthesis of the exopolysaccharide (Fig. 2). The absorption parameters did not change during the incubation period from 6 to 24 hours, and we observed simultaneous decreases in EPS accumulation and the OD of Congo red staining (*p*=0.015).

We observed changes in the dynamics of biofilm formation by the *S. aureus* strains isolated from the wounds of the Test Group patients after WBP but prior to surgery. After 4 and 6 hours of incubation, the OD values of the Congo red-stained eluates from the samples obtained after WBP were lower than those obtained prior to treatment (*p*=0.003). This finding indicates a decrease in biofilm polysaccharide production after complex WBP. The *in vitro* biomass production of the post-WBP isolates from the Test Group decreased 18 hours after treatment.
the reduction in polysaccharide production \((p=0.013;\) Fig. 3). The biofilm formation at 18 to 24 hours among the strains isolated immediately prior to surgery did not differ from that among the strains isolated at the time of hospital admission. The exopolysaccharide synthesis by the strains isolated after WBP, however, decreased. In addition, the Congo red OD values were lower after 48 hours of incubation than they were initially \((p=0.038;\) Fig. 3).

The dynamics of biofilm formation among the \(S.\ aureus\) strains isolated from the patients in the Control Group following WBP did not differ from those among the strains isolated from the same patients at the time of admission (Fig. 4).

We examined the differences in the dynamics of the graft-healing process and the frequency of graft loss during the post-procedural period (Figs. 5 and 6) by measuring biofilm formation among the strains isolated from loss grafts (Control Group) and at the moment of skin-graft healing (Test Group). The Congo red-absorption parameters of the isolates from the wounds that experienced graft loss were greater than those of the isolates obtained after complete graft healing \((p=0.037,\ p=0.004)\). This result indicates that graft loss is accompanied by a higher capacity for \(S.\ aureus\) to form biofilms. The \(S.\ aureus\) isolates from the wounds that experienced graft loss demonstrated a lower capacity for biomass formation and an increased capacity for exopolysaccharide accumulation at the beginning of the incubation period (2-4 hours). This finding was confirmed by lower crystal violet-extinction parameters for isolates from the wounds that experienced graft loss (Fig. 6). We observed higher biomass \((p=0.01)\) after 6 hours of incubation, although the OD values for crystal violet staining did not differ between the groups during the rest of the incubation time.
DISCUSSION

Experimental studies describe biofilm formation by different bacteria, including *Staphylococcus* species[23,24]. Bacteria attach to one another within several minutes and begin to develop the initial EPS within 2 to 4 hours. After beginning to form the EPS, the bacteria become increasingly tolerant to biocides within 6 to 12 hours. Within 24 hours, the bacteria develop mature biofilm colonies that are extremely resistant to biocides and shed planktonic bacteria. Therefore, the application of appropriate treatment at the early stages of biofilm formation is critical for biofilm management in chronic wounds.

UD treatment involves the removal of contaminated tissue and disrupts the formation of the biofilm in the wound by destroying the EPS[17,18]. The use of TNP, which involves the removal of wound exudates and the reduction of bacteria during WBP, prevents bacterial attachment[19]. The complex use of UD and TNP facilitates the formation of granulation tissue and further helps to promote the wound-healing process[13,14,15]. Our experience is that the combination of UD and TNP is an excellent method for WBP prior to skin grafting, in contrast to other standard treatment methods.

The results of our *in vitro* studies of biofilm formation by *S. aureus* strains isolated from chronic wounds support the hypothesis that the complex use of UD and TNP prior to skin grafting has a significant, positive effect on treatment outcomes. Treatment with UD and TNP resulted in a decreased capacity for *S. aureus* to form biofilms *in vitro*. The reduced capacity to form biofilms was a predictor of skin-grafting outcome; and the result was confirmed by the differences in OD values for Congo red staining measured at 2 to 18 hours in the Test Group and the Control Group. We detected higher crystal violet-absorbance parameters during the early stages of incubation (2-4 hours) in the patients with favourable skin-grafting outcomes compared with those in the patients with graft loss. The isolates from patients with favourable outcomes exhibited increased proliferation and decreased biofilm-forming capacity (Fig. 6). The absence of a thick layer of EPS did not prevent the influence of local factors for wound protection, and thus, *S. aureus* contamination in wounds treated with UD and TNP did not cause infection or prevent healing. Therefore, we propose that the presence of *S. aureus* strains with a low biofilm-forming capacity will not influence the process of graft fixation. On the other hand, the presence of *S. aureus* strains that quickly form biofilms will affect the contact characteristics of the wound bed and impede the process of graft fixation, resulting in graft rejection. Hence, the influence of *S. aureus* biofilm formation should be taken into consideration when choosing the methods for WBP.

The application of standard treatment methods did not alter the capacity of *S. aureus* to form biofilms. As a result, the
standard treatment methods did not achieve more favourable results in graft healing: in the Control Group, 20% of the patients experienced graft failure. Also, patients in the Control Group that had successful graft healing during the post-procedural period exhibited unstable graft signs; as a result, healing took longer in the Control Group than in the Test Group. Therefore, the conventional treatment of chronic wounds appears to be of little use in improving the outcome of skin grafts, and this notion is supported by other studies.

The complex use of UD and TNP did not influence the key stages of biofilm formation in vitro; these methods, however, directly influenced the pathophysiological mechanisms of chronic wounds. UD removes non-viable tissues and reduces bacterial load. TNP promotes healing via the removal of soluble healing inhibitors from the wound and thus increases tissue perfusion, inactivates capillary autoregulation, allows the proliferating cells to rest between cycles of cell division and produce new cellular components, stimulates angiogenesis and epithelialisation, and draws the wound edges closer together.

Overall, our isolation of etiologically significant *S. aureus* strains from chronic wounds and our determination of their capacity to form biofilms with 2 to 6 hours of incubation support the complex use of UD and TNP for WBP before surgical closure.

**Implications for Clinical Practice**

Studies of the capacity of *S. aureus* isolates to form a biofilm in vitro can influence the choice of methods for WBP prior to skin grafting. If *S. aureus* strains with an active capacity to form biofilms in vitro within 2 hours of incubation are isolated from a chronic wound during an initial bacteriological examination, then the patient is a good candidate for UD and TNP, because the bacterial strains in their wounds are likely to influence the healing of the graft. The influence of the thick layer of the main biofilm substance on the process of graft fixation should also be taken into account. Because *S. aureus* strains with a low capacity for biofilm formation, evidenced by EPS accumulation after 6 hours of incubation in vitro, do not influence graft fixation and healing, conservative methods of standard wound dressing will be effective for WBP in wounds harbouring those strains.

**Further research**

In the future, we will repeat these studies with increased numbers of patients in the experimental group and confirm the results in a practical setting. We are also studying the influence of different methods of wound treatment on biofilm formation by other etiologically important strains of bacteria, such as *Enterobacteriaceae*, *Enterococcus faecalis*, and non-fermenting gram-negative rod bacteria.

**CONCLUSION**

The biofilm-formation capacity of bacteria from chronic wounds can be used for choosing the best treatment method for WBP prior to skin grafting.