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In-situ characterization of the bacterial biofilm associated with Xeroform™ and Kaltostat™ dressings and evaluation of their effectiveness on thin skin engraftment donor sites in burn patients

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ABSTRACT

Biofilm forms when bacteria surrounded by an extracellular matrix aggregate on a surface. It can develop on many surfaces, including wound dressings; this can be particularly nefarious for burn patients undergoing skin grafting (autograft) for burn wound coverage as they often suffer from compromised immune system function. Autograft donor sites are particularly vulnerable to biofilm formation; as such, timely healing of these sites is essential. Our aim was to apply scanning electron microscopy to compare the efficacy of two types of wound dressings in preventing the formation of bacterial biofilm on burn patient skin graft donor sites. One dressing contained bismuth tribromophenate at a concentration of 3% which confers it bacteriostatic properties (Xeroform™). The other was an absorptive alginate calcium sodium dressing (Kaltostat™). Samples of each wound dressing, which were in contact with the skin graft donor site, were prepared for analysis under the scanning electron microscope (SEM) using an original method developed by our research group that aims to maintain the integrity of the biofilm microstructure. Samples prepared by this method were then analyzed using SEM, which allowed the characterization of biofilm and the evaluation of bacterial density on the studied dressing samples. To this day, this imaging technique has been rarely employed for dressing analysis and this is the first time that it is employed for *in situ* biofilm visualization for this particular application.

1. Background

Biofilms comprise clusters of bacteria coated by a matrix of extracellular polymeric substances (EPS) that may form on substrates such as a wound dressing [1]. Biofilm, which is typically thin and viscous in appearance, physically protects the bacterial microorganisms while conferring resistance to the host's immune response and conventional antimicrobial agents. The EPS matrix mainly consists of polysaccharides, proteins, nucleic acids and lipids. These components provide mechanical stability of biofilms by measuring their adhesion

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to surfaces and forming a three-dimensional polymer network that transiently interconnects and immobilizes biofilm cells [2].

Chronic wounds are defined as skin lesions that persist beyond 6 weeks, reflecting an alteration in the normal healing process [3]. According to scanning electron microscopy analyses, an estimated 60% of chronic wounds are populated by bacterial biofilm [4]. Indeed, one of the first clinical signs of an infected wound is a delay in the normal healing process. Delayed wound re-epithelialization may become a source of pain and functional discomfort and ultimately affects scar aesthetics and quality [5]. As such, the presence of a bacterial biofilm strongly compromises normal wound healing at skin graft donor sites, thereby also generating additional costs for the patient and healthcare system alike [6].

Skin grafting, or autografting, is an essential component of burn patient surgical treatment. It involves the harvest and transfer of skin from an intact part of the body to the debrided burn wound. Optimized donor site healing is extremely important in the burn patient population, as they often experience concomitant immune system dysfunction. To date, there is no consensus as to which type of wound dressing is most beneficial in terms of bacterial biofilm risk, pain control, scarring and final aesthetic appearance [7]. Interestingly, split skin graft donor sites also represent an excellent research model for general wound healing. A number of dressings and topical agents for donor site wounds are currently available and practices vary widely across centres. One commonly used dressing option is an alginate calcium sodium dressing (Kaltostat™). Alginates are intended to provide absorptive and mild hemostatic properties on contact with wound exudate and are biodegradable dressings derived from seaweed; they are also supposed to maintain a physiologically moist microenvironment that promotes wound healing. Fischer et al. [8], in their *in-vitro* study showed that despite the alginate origin or addition of antimicrobials, all dressings are able to reduce the concentration of the proinflammatory cytokines TNF-α and IL-8. The authors concluded that the enhanced binding affinity by microbial alginate may be interesting to support optimal conditions for wound healing [8].

Another commonly used dressing is a petrolatum-based fine mesh gauze containing 3% bismuth tribromophenate (Xeroform™). This dressing has been traditionally used in plastic and burn surgery for its antimicrobial and deodorizing properties as well its ability to maintain a moist environment for wound healing. Even though Barillo et al. [9], have shown that *in-vitro* the dressing with bismuth tribromophenate (Xeroform™) does not show antibacterial activity [9], this study shows that clinically Xeroform dressings show the opposite, an antibacterial activity superior to the other dressing (Kaltostat™), commonly used in our burn unit.

The scanning electron microscope (SEM) is a powerful tool with a magnification up to 500,000×. Its operation is based on the interactions between the studied surface and the electrons. It is an important technique in studying the interactions between biomaterials and dressings that we use on the human body. In 2010, researchers worked on a selection of non-occlusive and non-adhesive dressings to determine their effect on bacterial biological burden. They tested the growth of bacterial biofilm by several techniques including scanning electron microscopy [10]. Another group of researchers used debridement with traditional dressings to remove necrotic tissue and bacterial biofilm. Removal of the biofilm was in part determined using the SEM [11]. Despite being a state-of-the-art tool for biomaterial surface characterization, there are no studies employing SEM for wound dressing analysis *in vivo* on human subjects have been reported in the literature.

In this prospective study, we compared these two commonly used donor site dressings in the burn population. Using SEM, Kaltostat™ and Xeroform™ dressing samples derived directly from actual burn patient donor sites were analyzed for bacterial biofilm density and then compared.

The main aim of this research is to assess the utility of scanning electron microscopy as technique for biofilm characterization on Kaltostat™ and Xeroform™ dressings in contact with skin graft donor site wounds in human burned patients.

2. Methods

2.1. Study participants

Our team adhered to the rules and regulations of its hospital center ethics board (Centre Hospitalier de l'Université de Montréal Ethics Committee), which approved this study.

We included prospectively in the study, patients aged 18 or more, admitted for burns of at least 10% of their total body surface area (TBSA) and requiring surgical treatment of their burn injuries (with autografting of more than 200 cm^2) were prospectively included in this study. Only patients giving consent (or *via* a surrogate when the patient was unable to provide consent personally) were recruited. Minors (aged less than 18 years), adult patients with less than 10% TBSA burns, and those refusing consent were not included in this study.

2.2. Study design

During the burn debridement and skin graft coverage procedures, the skin grafts were taken from the same anatomical region of a given patient by the surgeon using a pneumatic or electrical dermatome set to a thickness of 0.254 mm in order to create standardized wounds. The patients received a standard non-bacteriostatic alginate dressing (Kaltostat™) at a thin skin graft donor site and a dressing with gauze dressing with bacteriostatic properties (3% Bismuth Tribromophenate) commercially known as Xeroform™ at a second adjacent donor site of the same size.

At 5, 10 and 15 days postoperatively, samples of the respective dressings were taken, and their surfaces evaluated qualitatively and semi-quantitatively for biofilm by SEM. At the same three afore mentioned time points, the donor site was analyzed in terms of the reepithelialization time, discomfort, pain and appearance of the wound. In this paper, only the data obtained in the context of the SEM are presented.

2.3. *Scanning electron microscopy sample preparation and analysis*

SEM images were acquired using a Quanta FEG 450 ESEM (FEI Company) in high vacuum mode with working distance 5 mm and accelerating voltage 5 kV.

For SEM analysis at the three time points, 1 cm^2 samples of each dressing type were taken and immediately fixed in a 2% solution of glutaraldehyde in sodium cacodylate buffer at pH 7.3 and stored at 4 °C for at least 24 h. The following steps were then done to prepare dressings and wound biopsies samples for SEM analyses:

1) Samples were dehydrated by increasing concentrations of ethyl alcohol (30%, 50%, 70%, 80% and 100%), for 15 min each (time could be adjusted in relation with the sample size).
2) A drying step was conducted at the critical point with 100 µl of hexamethyldisilazane (HMDS) which was placed on each sample then left under a hood until completely dry.
3) Prepared samples were then platinum-coated just before being analyzed in conventional SEM.

The rationale behind the above technique was to need to preserve the structure of the sample by reducing potential damage caused by the preparation, thereby maintaining the integrity of the natural state of the sample as much as possible.

Two negative controls were analyzed: samples of the two dressings (Kaltostat™ and Xeroform™) before their application were used to observe the porosity and the general appearance of the surface of the respective dressings. For each dressing, three low magnification images (400×) taken at different areas of the sample were recorded and used to identify areas of interest. For each area of interest, 3000× and 6000× images were also taken and analyzed. For each type of dressing we compared information about the dressing surface, the cell count, the presence/absence of a biofilm, and its density. As for the analysis of the images, three observers firstly determined the observable elements in all the images. Only the parameters common to the 3 observers were kept for the analysis. These parameters (Table 1) were compared using the high-magnification images, namely 9 images per sample (3000× and 6000×), either with a count of bacteria developed by our research team [12], when the parameter lent itself to it, or with Van Heerden's semi-quantitative biofilm scale (0 = no biofilm coverage, 1 = small area of biofilm coverage, 2 = medium area of biofilm coverage, and 3 = large area of biofilm coverage) [13]. In this way, the whole surface area of each wound dressing sample was examined at a constant magnification, which permitted grading of the area of biofilm coverage on the sample by each observer. It is possible to consult images of samples at each grade of biofilm in van Heerden et al.'s [13] publication.

3. Results

We prospectively included 10 patients in the study. Patient's demography, percentages of TBSA and location of the donor sites (usually on the thigh and more rarely on the back) are summarized in Table 2. Patients were aged between 24 and 76 years with a mean age of 49 years and among the 10 patients recruited, eight are male.

3.1. *Negative control*

Before beginning patient recruitment, we started by developing the sample preparation technique for scanning electron microscopy. We have started by analyzing the negative controls, the dressings before use, to visualize the microstructure of the fibers that form these two different dressings and later compare them with the samples taken from the donor sites of patients. This allowed us to establish the right parameters of SEM analysis, the ideal magnifications for visualization of dressing fibers, and the peculiarities of these dressing fibers (see box in Fig. 1) which could lead to confusions once these dressings are covered with bacteria or other cellular debris after contact with the donor site of the patient. Fig. 1 displays images of the control samples, corresponding to the two wound dressings in their unused states.

3.2. *Recruitment of 10 patients, sample collection and analyses*

The images and charts presented in Fig. 2 display a clear trend regarding the predominance of bacteria and the presence of bacterial biofilm in the Kaltostat™ wound dressings compared to the Xeroform™ wound dressings. Using the grading system described previously in the *Material and Methods* section, a score was assigned for each sample after analyzing approximately 9 *highly magnified* images (3000× and 6000×).

Table 1 – Scoring system using semiquantitative scale of biofilm surface for Van Heerden (A), biofilm thickness for Danino (B), and cellular presence (C).

Grade	Scoring system (Van Heerden) for biofilm surface (A)	Scoring system (Danino) for biofilm thickness (B)	Cellular presence (C)
0	No biofilm coverage	No biofilm coverage	No cells
1	Small area of biofilm coverage	Thin, in which the extracellular matrix does not cover bacteria	Low cellular density
2	Medium area of biofilm coverage	Thick biofilm coat	Intermediate cellular density
3	Large area of biofilm coverage	Very thick biofilm coat	High cellular density

Table 2 – Patient's demography, percentages of TBSA and location of the donor sites for the ten patients.

Patient	Age	Sexe	TBSA (%)	Donor sites	
				Absorptive alginate (*Kaltostat*™) wound dressings	Bacteriostatic (*Xerofom*™ wound dressings
Go01Je	57	M	38	Right thigh	Back
Jo2Mi	60	M	13	Right thigh, posterolateral	Left thigh, anteromedial
Pe3Sa	28	M	25	Left thigh, posterolateral	Left thigh, anteromedial
Ha4Br	51	M	11	Right thigh and left thigh	Right thigh and left thigh
Ve5Ch	60	F	14	Right thigh, medial	Right leg
Po6Ra	76	M	10	Left thigh, medial	Left thigh, lateral
Bo7Sy	49	F	10	Left thigh, lateral	Left thigh, medial
Le8Je	51	M	65	Upper back	Lower back
Ma9To	24	M	14	Left thigh	Left thigh
Ha10St	39	M	35	Back	Back

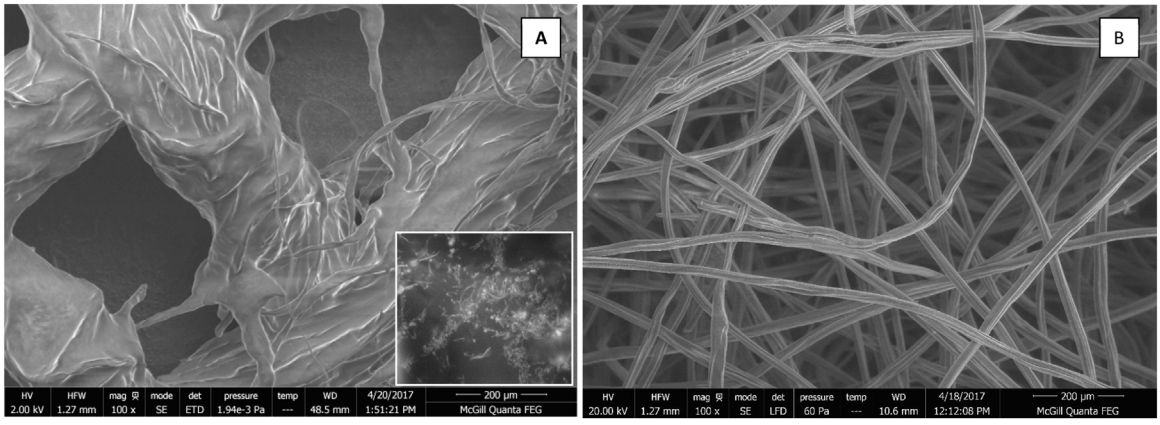

Fig. 1 – Control samples for occlusive bacteriostatic (Xerofom™) (A) and absorptive alginate (Kaltostat™) (B) dressings. In the box of (A) it is possible to observe the microstructure of surface fibers in the occlusive bacteriostatic wound dressings which can easily be mistaken for bacterial presence.

Fig. 3 shows some of the most representative SEM images, images taken on days 5, 10 and 15 after the surgery, of sample collection for a patient who participated in the study. These pictures are also very representative for the 10 patients included to the pilot prospective study from the point of view of the tendency of the preponderance of bacteria and bacterial biofilm for Kaltostat™ *versus* Xeroform™ dressings.

There are different types of classifications of bacteria. The classification of Linnaeus makes it possible to distinguish different levels: the reign, the branch, the family, the genus and the species. Another classification, frequently used, corresponds to their reaction in contact with the Gram stain; it is a method to differentiate bacteria based on their staining capacity which varies according to their respective wall compositions. Many bacteria have affinity for the environment of human skin. These microorganisms, which comprise the skin microbiome, belong to various families such as staphylococci, streptococci, Gram-negative bacilli, and gonococci among others. These different types of bacteria were easily identifiable in SEM images, both in isolated forms and in clusters within biofilm. This was especially true for the Kaltostat™ dressing. For example, it was possible to observe cocci-type bacteria establishing biofilm, in Fig. 3. Bacilli-type biofilm (patients Go01Je, Jo2Mi and Ve5Ch) and streptococci (more predominant than baccili type) were also observed. All these types of bacteria were detected for both dressings, the main difference being that they were highly visible in the Kaltostat™ dressing and were less notable in both quantity and frequency in the Xeroform™ dressing, as evidenced by the two different scores shown in Fig. 2.

Fig. 4 shows two SEM images of the Kaltostat™ dressing which were colourized using the program MountainsMap® SEM Topo. These images can attest to the presence and extent of bacterial biofilm in a lower magnification view image (A) where bacterial biofilm is coloured in brown and wound dressing fibres are coloured in blue. Furthermore, a higher magnification view image (B) shows bacterial clusters (coloured in brown) as well as the extracellular polysaccharide network (EPS matrix, coloured in green) which is partially surrounding the bacteria. Table 3 presents the different type of bacteria observed on the two dressings analyzed for the 10 patients under study, and average van Heerden and Danino scores (taken from days 5, 10 and 15), for Xeroform™ and Kaltostat™ wound dressings used on donor sites.

These results demonstrated that samples of wound dressings in contact with burn patient skin graft donor sites of burn patients can be successfully characterized using

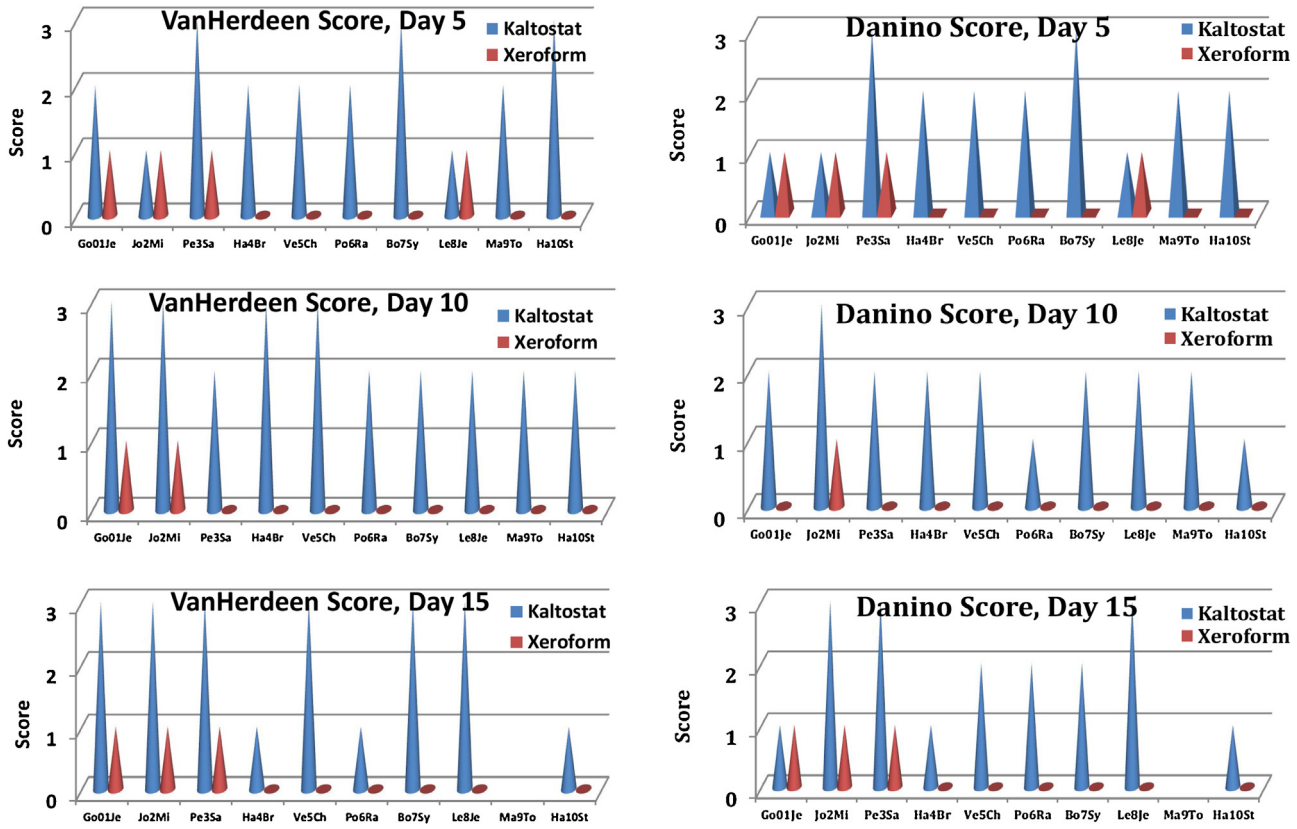

Fig. 2 – Van Heerden and Danino scores for 3 sample collections of occlusive bacteriostatic (Xerofom™) and absorptive alginate (Kaltostat™) wound dressings, taken from the 10 studied patients.

scanning electron microscopy. Indeed, bacteria and bacterial biofilm can be easily detected with this technique combined to the method of sample preparation which was employed here.

4. Discussion

Due to the damage of normal skin in affected parts of their body, burn patients are particularly vulnerable to bacteria and consequently biofilm. The most efficient way to remove biofilms is through the use of mechanical techniques, including debridement and effective wound care. Wound dressing fibers, such as polyacrylate fibers, have been proven to be successful in affecting biofilm architecture by disrupting the biofilm matrix [14].

A very effective two-day postoperative bandage can become harmful a week later. Exudative wounds therefore need an absorbent dressing such as alginates (Kaltostat™), whereas dry or dehydrated wounds benefit from the provision of moisture such as wet compresses, hydrogels or occlusive dressings (Xeroform™). Occlusive dressings also have the advantage of inhibiting bacterial growth and creating an impermeable barrier to exogenous bacteria which migrate from the external environment to the wound [15]. As early as 1995, Hansbrough and his research team showed that for skin graft, occlusive dressings are much more effective compared to wet dressings that do not possess bacteriostatic properties [16].

The risk of chronic wound infection has sparked interest in innovative dressings with bacteriostatic properties. In 1991, researchers evaluated the effectiveness of different dressings used on donor site in 30 patients with respect to wound healing, pain, infection, and cost. Despite being less comfortable than other dressings, the newer compresses with bacteriostatic properties had the shortest average healing time and were associated with lower infection rate as well as cost per patient [17]. In the literature, it has been proven that the bacteriostatic properties of this wound dressing preserve the normal healing process while minimizing the risk of bacterial infection. On the other hand, its potential to prevent the development of bacterial biofilm has been poorly documented, especially in the setting of direct visualization of bacterial biofilm using an advanced modern technology, as the scanning electron microscopy.

Scanning electron microscopy is a technique that requires a minimum of time for sample preparation and allows scanning a surface on a scale of hundreds of microns to millimeters while having the advantage of being able to mount the magnification very quickly in the area of interest identified at low magnification. In several articles published by our research group, we have already demonstrated the effectiveness of conventional SEM use in the field of breast

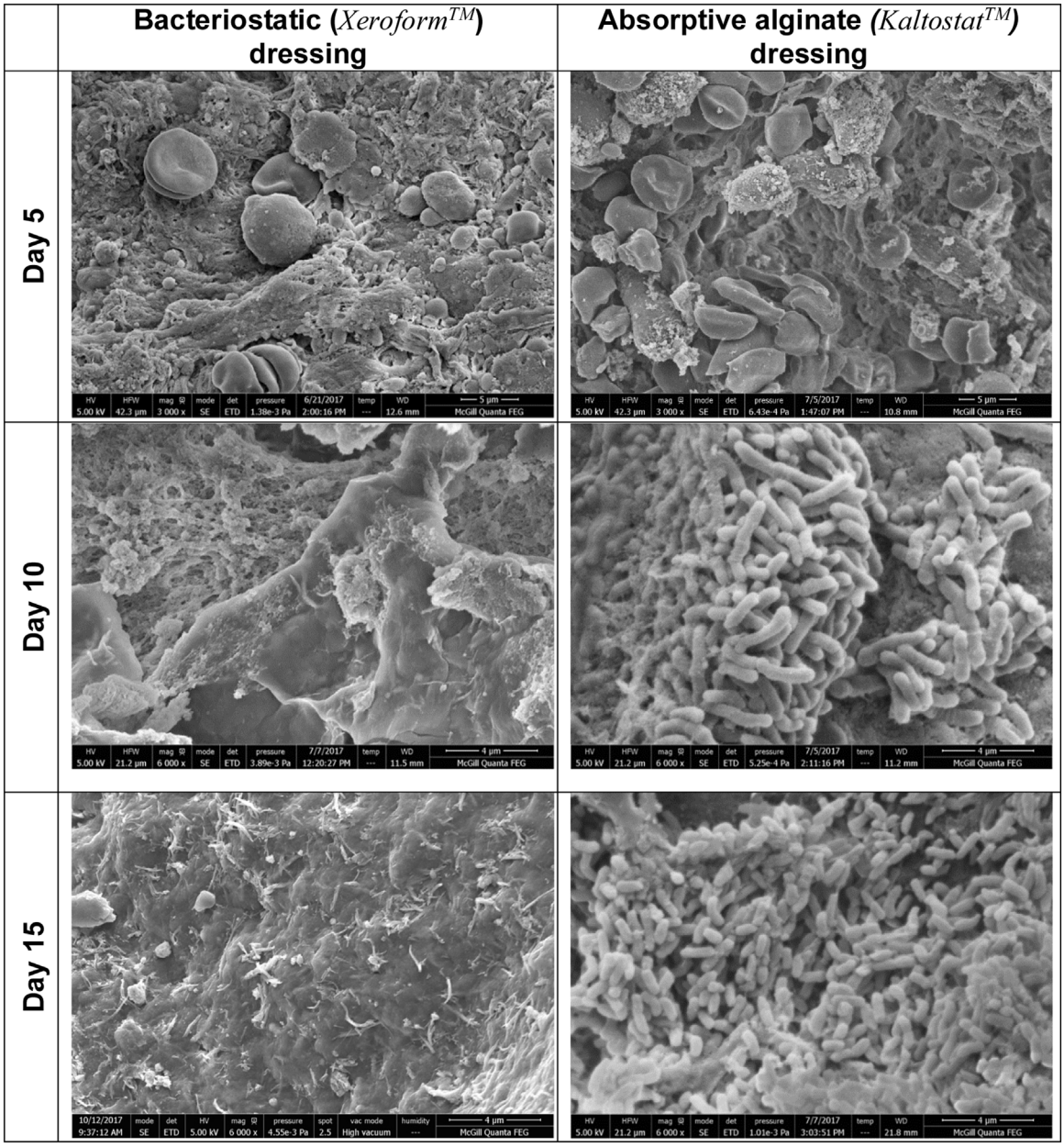

Fig. 3 – Jo2Mi patient: donor site on the right thigh (Kaltostat™ wound dressing in posterolateral and Xeroform™ wound dressing in anteromedial).

reconstruction [18–20]. In the 2013 study, the authors compared two specific SEM procedures: conventional (high vacuum) and environmental (wet) and they ascertained that the conventional mode was superior for the purpose of breast prosthesis and capsule analysis based on the obtained image quality and reduced artifact caused by sample preparation. In this study, we chose to employ the conventional mode.

In the past, scanning electron microscopy has been used successfully for biofilm analysis [21]. Indeed, the SEM was used to evaluate *in situ* bacterial colonization of intravenous and intra-arterial catheters as early as 1984 [22]. However, standard SEM requires biological samples to be critical point dried and metal-coated prior to observation; since biofilms consist primarily of water, dehydration of the sample may alter biofilm structure. When coated with hexamethyldisilazane (HMDS), which is an organic compound, biological samples do not require dehydration or metal-coating [23,24]. In previous studies, hexamethyldisilazane (HMDS) has been used as a drying agent for specimen preparation for scanning electron microscopy, whether for visualization of retinal tissue, pollen, or bacterial surface colonization on coal [25–27]. After testing several methods (classical CPD-critical point dryer, ionic liquids and HMDS), we have chosen to use the HMDS product because of the quality of the results and integrity of the biofilms and bacteria seen under the SEM.

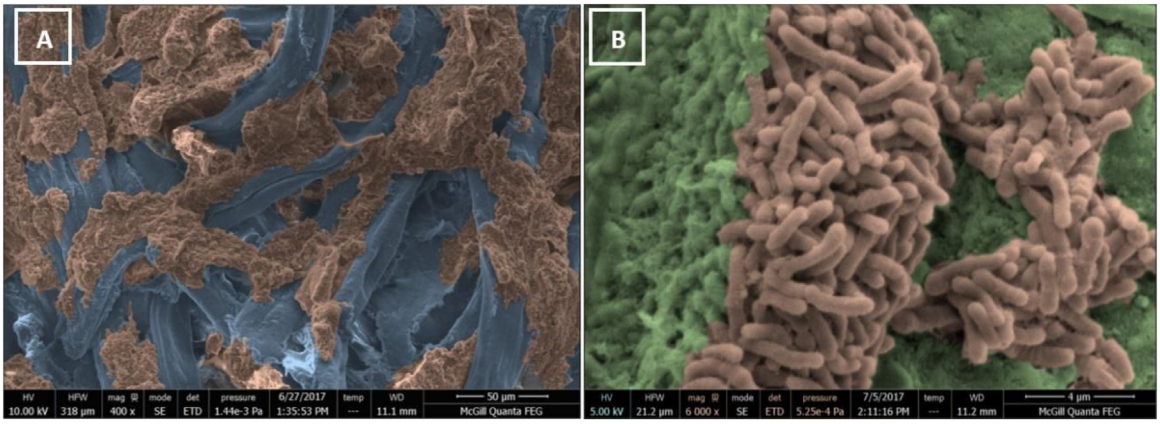

Fig. 4 – Colourized SEM images of the Kaltostat™ wound dressing (MountainsMap® SEM Topo program). The first image (A) shows, at a lower magnification, the extent of bacterial biofilm; bacterial biofilm is represented in brown and wound dressing fibers are represented in blue. The second image (B) demonstrates, at a higher magnification, clusters of bacteria which are represented in brown as well as the polysaccharide network (EPS matrix) which is represented in green. The EPS is partially enveloping the bacteria in order to form the biofilm. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

Table 3 – Average van Heerden and Danino scores (taken from Days 5, 10 and 15) for occlusive bacteriostatic (Xerofom™) and absorptive alginate (Kaltostat™) wound dressings donor sites.

Patient	Average van Heerden score		Average Danino score	
	Absorptive alginate (*Kaltostat*™) wound dressings	Bacteriostatic (*Xerofom*™) wound dressings	Absorptive alginate (*Kaltostat*™) wound dressings	Bacteriostatic (*Xerofom*™) wound dressings
Go01Je	2.67	1	1.33	0.67
Jo2Mi	2,33	1	2.33	1
Pe3Sa	2,67	0.67	2.67	0.67
Ha4Br	2	0	1.67	0
Ve5Ch	2.67	0	2	0
Po6Ra	1.67	0	1.67	0
Bo7Sy	2.66	0	2.33	0
Le8Je	2	0.33	2	0.33
Ma9To	2	0	2	0
Ha10St	1.33	0	1.33	0

In this study, we demonstrated that SEM can be employed for *in situ* biofilm analysis on wound dressings, a purpose for which this technique has never been employed before. For the first time, we used SEM to study biofilm on wound dressings in order to compare the ability of two different dressing types in hindering biofilm formation. Our results demonstrate the high efficiency, utility and versatility of SEM. Indeed, we have demonstrated the efficiency of SEM in the characterization of *in situ* biofilm by confirming the results of other studies, which point towards the ability of bacteriostatic wound dressings to prevent and delay biofilm formation in skin graft donor sites. Using SEM, it is possible to visualize the bacterial biofilm *in situ*, to analyze its extent and to count the bacteria present at magnifications ranging from 3000–5000 times the natural size [28]. This technique allowed us to observe that while donor sites covered with the commonly used wound Kaltostat™ often became breeding grounds for microorganisms, the dressing with bacteriostatic properties (Xeroform™) hindered bacterial proliferation and accelerated wound healing. Occlusive bacteriostatic dressings were therefore more effective in managing the content of exudate in contact with the wound. These observations were validated by those presented in the earlier mentioned studies by Chattopadhyay et al. [15] and Hansbrough et al. [16]. In other words, bacterial biofilm formation was more pronounced in the wound dressing without bacteriostatic properties (Kaltostat™).

5. Conclusion

In this study, we used scanning electron microscopy to visualize the presence or absence of biofilm *in situ* and evaluate the bacterial density on two types of dressings.

The analysis and results obtained for the 10 patients studied revealed that bacterial biofilm is less likely to form in wound dressings with bacteriostatic properties than in wound dressings with no bacteriostatic properties such as the *Kaltostat*™ compresses. SEM is undoubtedly an immensely valuable tool for the analysis of wound dressing samples and permits researchers to visualize and characterize bacterial biofilms. Provided that the preparation technique is appropriate, the potential for SEM to study bacteria and biofilms is wide-ranging. The numerous possible applications of this technique in biomedical research merit further exploration.

Disclosure

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.burns.2019.02.024.

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