

A New Acid-oxidizing Solution: Assessment of Its Role on Methicillin-resistant *Staphylococcus aureus* (MRSA) Biofilm Morphological Changes

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Wounds 2015;27(10):265-273

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Disclosure: Dr. D'Atanasio, Ms. Capezzone de Joannon, Ms. Mangano, Mr. Milanese, and Dr. Tongiani are employed with Angelini Research S.p.A.; Dr. Meloni is chief executive officer of VitroScreen Srl; and Dr. Giarratana is employed by APR Applied Pharma Research S.A.

Abstract: *Objective.* Biofilms represent a key challenge in the treatment of chronic wounds, as they are among the main reasons for delays in chronic wound healing. This in vitro study was aimed at evaluating the activity of a new acid-oxidizing solution (AOS) on biofilm formation. Acid-oxidizing solution contains free chlorine species with stabilized hypochlorous acid in high concentration (> 95%) and is characterized by acidic (pH < 3) and super-oxidizing (Redox > 1000mV) features. *Materials and Methods.* A 3-dimensional in vitro model of reconstructed human epidermis was used to compare the activity of AOS vs 2 reference products (RP) containing betaine and polyhexanide (RP1) and sodium hypochlorite and hypochlorous acid (RP2). Different approaches were used to assess the prevention and eradication of methicillin-resistant *Staphylococcus aureus* biofilm by the study products. Xylitol and chlorhexidine were used as positive controls. The activity of the study products on the biofilm structure was evaluated analyzing the ultrastructural modification by scanning electron microscopy, while skin compatibility was assessed on noncolonized tissues measuring the metabolic activity of the cells. *Results.* In all experiments, AOS showed to be active on the biofilm matrix, modifying its structure and allowing bacterial release from the matrix. In all experiments, no cytotoxicity was observed in the tissues treated with the product suggesting a good compatibility of AOS with skin tissues. Reference product 1 affected the biofilm, suggesting a disruption effect; RP2 was slightly less active than AOS in modifying the biofilm structure. *Conclusion.* Treatment with AOS affects biofilm by modifying its structure and therefore facilitating local bacteria accessibility to bactericidal agents, with consequent potential clinical benefits in the treatment of chronic wounds.

Key words: acid-oxidizing solution, biofilm, bacterial infections, chronic wound treatment

Biofilms represent a model of bacterial growth that allows both the survival of microorganisms in hostile environments and the colonization of new niches through the dispersal of bacteria.^{1,3} Biofilms are associated with a self-produced hydrated matrix of extracellular polymeric substances (EPS) comprised of polysaccharides, proteins, lipids, and extracellular DNA.^{4,6} This matrix confers structure and protection to the complex biofilm community that becomes resistant against changes of the environmental conditions.⁷

Besides surface association, aggregation, and production of a matrix, there are several differences between biofilm-associated microorganisms and their free-floating planktonic counterparts. First of all, biofilm-included bacteria can be inherently more resistant to innate immune responses as well as the activity of antibiotics and other antimicrobial treatments since the biofilm may shield colonies from endogenous defense mechanisms and can prevent diffusion of locally applied products because of its structural and physicochemical characteristics.⁸⁻¹⁰ Moreover, the metabolic characteristics of bacteria included in a biofilm are different from those of planktonic microorganisms since the complex structure of a biofilm tends to create niches in which metabolic cooperation often occurs. Finally, the closed structure of biofilm favors the acquisition of new genetic characteristics within the included population, increasing the onset of resistance. The overall result is that biofilm bacteria can be up to 1,000-fold more resistant to treatment than the same organisms grown planktonically, and can mediate infections that may have different characteristics from those caused by free-floating bacteria.¹¹

Biofilm formation is often associated with failure of acute wounds to proceed through the normal wound healing process which, clinically, may result in a slow and difficult wound healing process, or for the wound to be stalled in a chronic condition.¹² Bacterial behavior in chronic infections shows aggregation and, in most cases, attachment to a surface: this is the first step of a biofilm formation. Chronic wounds, such as diabetic foot ulcers, venous ulcers and wounds of different etiologies have a large impact on public health, and complications such as biofilm formation contribute to increasing the clinical and financial burden for their management. In fact, in the treatment of clinical infections characterized by biofilm development, standard antibiotic therapy is only able to eliminate mainly the planktonic cells, leaving the sessile forms still able to disseminate at the end of the therapy.

KEYPOINTS

- Biofilm formation is often associated with failure of acute wounds to heal normally.
- In the treatment of clinical infections characterized by biofilm development, standard antibiotic therapy is only able to eliminate mainly the planktonic cells, leaving the sessile forms able to disseminate.
- The aim of this study was to assess the effects of acid-oxidizing solution vs reference products on biofilm formation and eradication.

A new acid-oxidizing solution (AOS) for wound management has been developed as a stable super-oxidized solution prepared using a patented nanocoated electrolyzing unit. This solution contains free chlorine species with stabilized hypochlorous acid (HClO) in high concentration (> 95%) and is characterized by acidic (pH < 3) and super-oxidizing (redox > 1000mV) features.

In this nonclinical study, the activity of AOS on *in vitro* biofilm formation and eradication models was evaluated and compared to that of 2 marketed reference products (RP): RP1, containing betaine and polyhexanide, and RP2, containing hypochlorous acid and sodium hypochlorite.

The aim of the presented work was to assess the effects of AOS vs reference products on biofilm formation and eradication using *in vitro* experimental models based on 3-dimensional reconstructed human epidermis.¹³

Three different experiments were conducted: in the first 2 the prevention of biofilm formation was evaluated starting the treatments with the study products before bacterial colonization (time = 0) or 24 hours after bacterial inoculum. In both cases, cleansing with the study products was repeated at 24 hours (first experiment only), 48 hours, and 72 hours after bacterial deposition. In the third experiment, the eradication of biofilm was measured when starting treatments with the study products at 48 hours after bacteria deposition. This corresponded to the time of the mature biofilm formation, and to repeated cleansing with the study products at 52 hours and 72 hours from the inoculum.

Materials and Methods

In this study, the following study products were evaluated: RP1, containing purified water, undecylenamidopropyl betaine 0.1%, polyaminopropyl byguanide

(polyhexanide) 0.1%, pH 6-8; RP2, which is electrolyzed water (redox ~850 mV) plus sodium chloride, and also containing sodium hypochlorite and HClO (approximately 50%:50%) as ancillary substances, pH 6.2-7.8; and AOS, comprised of electrolyzed water characterized by acidic pH (<3) and high redox potential (>1000mV), and also containing free chlorine species with stabilized HClO in high concentration (>95%) as an ancillary substance. Saline solution was used as a negative control, and xylitol 5% and chlorhexidine 1% were used as positive controls in the experimental prevention and eradication models, respectively.^{14,15}

Reconstructed human epidermis (RHE) (The SkinEthic, RHE/S/12; Episkin, Lyon, France) of 0.5 cm² is a fully differentiated epidermis formed after 17 days of air-lift culture of normal human keratinocytes from skin biopsies in a chemically defined medium. The model reproduces epidermal morphology and has been fully characterized.^{16,17} The good barrier function, batch reproducibility, and low variability in terms of permeability for probe molecules make this model useful to predict the likely behavior of new products in humans.

Media and culture conditions. Reconstructed human epidermis cells were cultured according to the manufacturer's instructions. The cells were then removed from the agarose nutrient solution under a sterile airflow cabin. The inserts were rapidly transferred to 6-well plates previously filled with the provided growth medium (1 mL/well) (SkinEthic Growth Medium Episkin, Lyon, France) at room temperature and incubated at 37°C in a 5% CO₂ atmosphere in conditions of saturated humidity. Tissues were maintained in growth conditions for 17 days; the day before the experiment they were moved into maintaining medium without antibiotics (SkinEthic Maintenance Medium Episkin, Lyon, France).

Preparation of MRSA inocula and counts. The MRSA strain ATCC 33591 (LGC Standards, Milan, Italy) was used for all the experiments. After an overnight culture on nutrient agar (Nutrient Broth 70123-500G and Agar 20767.298 VWR, Sigma-Aldrich, St. Louis, MO), a few colonies were resuspended in phosphate buffered saline to obtain a concentration of about 4 x 10⁷ cfu/mL (Spectrophotometer OD 600 nm, Infinite 200 Pro, Tecan, Männedorf, Switzerland). The inoculum on the cells consisted of an apical addition of 50 µL to achieve the final concentration of 2x10⁶ cfu/tissue per well.

Bacterial counts were performed onto nutrient agar

KEYPOINTS

The following study products were evaluated:

- Reference product (RP) 1, containing purified water, undecylenamidopropyl betaine, and polyaminopropyl biguanide;
- RP2, containing electrolyzed water (redox ~850 mV), sodium chloride, sodium hypochlorite, and hypochlorous (HClO) acid as ancillary substances; and
- AOS, comprised of electrolyzed water characterized by acidic pH (<3) and high redox potential (>1000mV), and also free chlorine species with stabilized HClO in high concentration (>95%) as an ancillary substance.
- Saline solution was used as negative control, and xylitol 5% and chlorhexidine 1% were used as positive controls.

plating the appropriate 10-fold dilution of the different bacterial suspensions. After 1 day of incubation at 37°C, the colonies were counted visually.

Biofilm Prevention and Eradication Models

Biofilm prevention. The surface of tissue samples measuring 0.5 cm² were gently scraped and colonized with 50µL (2x10⁶ cfu/tissue) of MRSA ATCC 33591. Tissues were immediately (experiment I), or after 24 hours from inoculum (early bacterial colonization, experiment II), treated with 30 µL of each study product (ie, RP1, RP2, AOS) directly, uniformly, and topically on the epidermis. Subsequent treatments with the test items were performed at 24 hours (experiment I only), and 48-72 hours, without product removal. Tissues were harvested after 4 hours for all experiments; 5% xylitol and saline solution were applied as positive and negative controls respectively.

Biofilm eradication. The surface of tissue samples measuring 0.5 cm² were gently scraped and colonized with 50µL (2x10⁶ cfu/tissue) of MRSA ATCC 33591; after 48 hours from inoculum, corresponding to the mature biofilm formation, tissues were treated with 30 µL of each study product directly, uniformly, and topically on the epidermis, without product removal. Treatment was repeated at 52 hours and 72 hours. Tissues were harvested after 4 hours. Chlorhexidine 1% and saline solution were applied as positive and negative controls respectively.

Skin compatibility metabolic activity assay. The alamarBlue metabolic activity assay (In Vitro Toxicology Assay Kit, Resazurin based, Sigma-Aldrich, St. Louis,

MO) is based on a water-soluble dye that yields a fluorescent signal which undergoes a colorimetric change when incubated with metabolically active cells.

Tissues were placed into the medium containing 10% of resazurin. After 2 hours at 37°C in a CO₂ atmosphere at 5%, 100 µl aliquots of the medium were collected from each well and measured by spectrophotometer monitoring the fluorescence at excitation/emission = 560/590 nm. The metabolic activity assay percentage reduction (residual vitality) was calculated with respect to untreated tissues.

Scanning electron microscopy imaging. The cells were processed for scanning electron microscopy (SEM) imaging immediately after the acquisition of the confocal images. They were fixed with glutaraldehyde 1.2% in sodium cacodylate 0.1 M for 1 hour, washed 3 times with sodium cacodylate 0.1 M for 10 minutes, and postfixed with osmium tetroxide (OsO₄) 1% in sodium cacodylate 0.1 M for 1 hour. After removing the OsO₄ solution and rinsing twice with bidistilled water, the samples were gradually dehydrated by means of an ethanol series and then dried overnight with hexa-

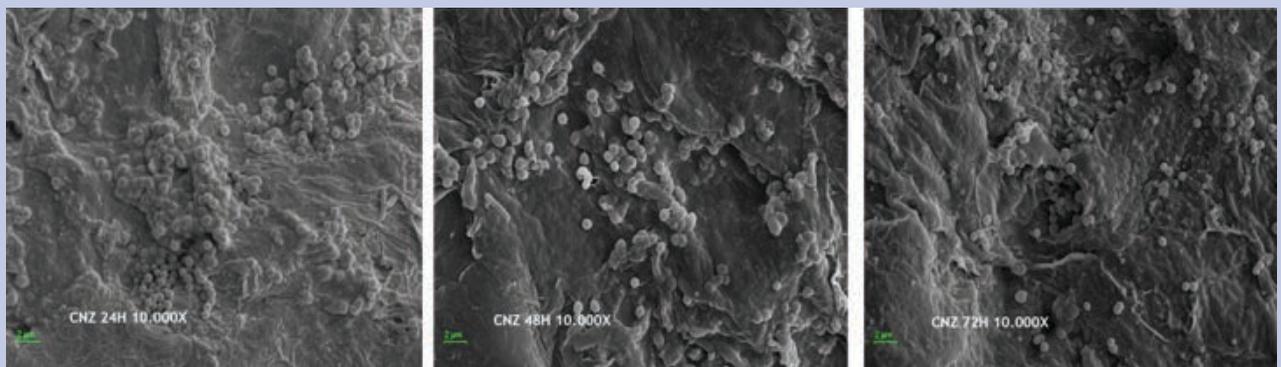


Figure 1. Scanning electron microscopy imaging analysis. Scanning electron microscopy performed on colonized control at 24 hours, 48 hours, and 72 hours. CNZ = colonized control

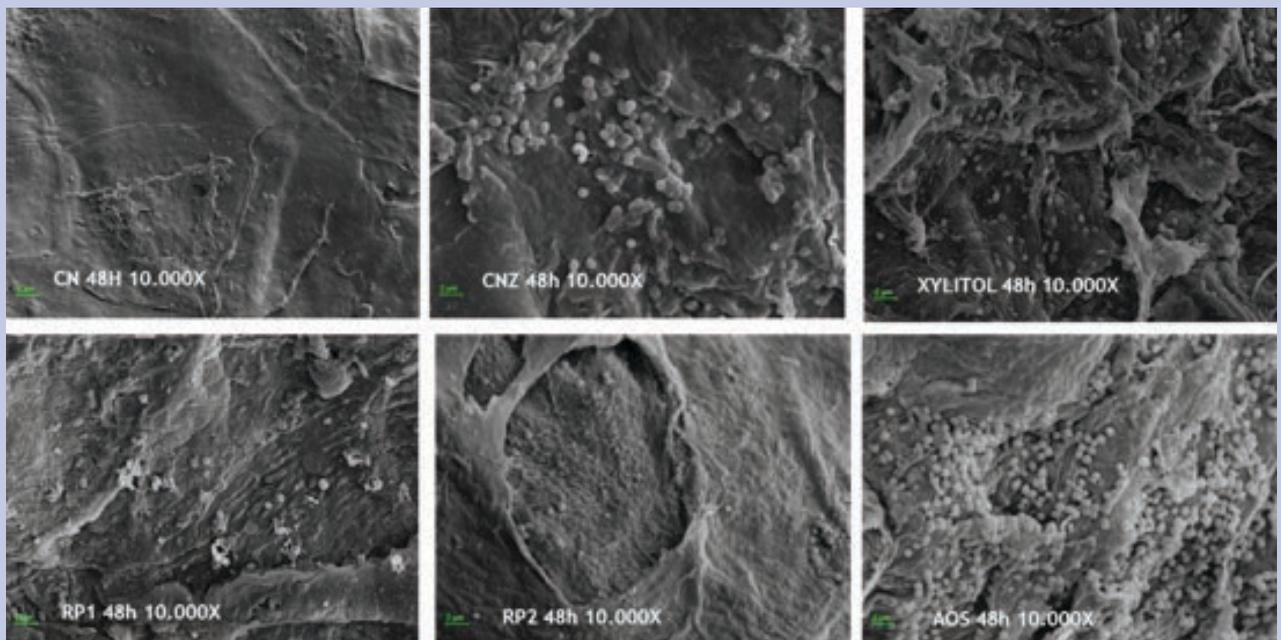


Figure 2. Scanning electron microscopy imaging analysis. Biofilm prevention experiment I. Tissues harvested 4 hours after treatment at 48 hours with reference product (RP) 1, RP 2, and acid-oxidizing solution compared with the controls. CN= noncolonized; CNZ= colonized; RP1: reference product 1; RP2: reference product 2; AOS: acid-oxidizing solution

methyldisilazane. All reagents were purchased from Electron Microscopy Sciences (Hatfield, PA). Once dried, the samples were sputtered with gold (Polaron E5100 Sputter Coater, Quorum Technologies, Ashford, Kent, UK) and the images were acquired at 1-5 kV using a field emission gun SEM (Sigma, Zeiss, Oberkochen, Germany) with a secondary electron detector.^{18,19} All images are shown at a 10,000x magnification.

Results

Time course of biofilm formation. Formation of biofilm following MRSA deposition was analyzed at 24 hours, 48 hours, and 72 hours (Figure 1). At 24 hours, the MRSA colonies were mostly enclosed in the EPS matrix. At 48 hours, the structure of the biofilm was clearly visible and bacteria were integrated in the EPS matrix with still visible fimbriae. At 72 hours, the MRSA colonies were shrouded in a dense and mature glycocalyx; a few single cells could be observed on the surface, ready to detach from the biofilm and colonize a new niche.

Biofilm prevention experiment I. At 48 hours from the inoculum in the colonized untreated control, the biofilm structure could be distinctly appreciated with bacteria clearly encapsulated into the EPS mucosal

polymeric matrix. In this test, the study products were applied 4 times every 24 hours starting at $t = 0$. Treatment with RP1 inhibited biofilm formation by eradicating the bacteria. On the contrary, after treatment with RP2, biofilm was still present but showed a less dense and less compact structure compared with the control. Treatment with AOS acted directly on the biofilm matrix determining the release of the majority of bacteria from the matrix with planktonic bacteria visible on the surface of the matrix. In the case of treatment with xylitol, the positive control, biofilm was still present but showed a different morphology from the colonized control with a less compact matrix (Figure 2).

Biofilm prevention experiment II. In this series of experiments, washings with saline and study products started at $t = 24$ hours and were repeated 2 times every 24 hours up to 72 hours. At 48 hours from inoculum bacteria in the colonized untreated control were completely encapsulated in the biofilm. With RP1 treatment, the biofilm was clearly in a dissolution phase; the visible cocci showed a smaller size compared to those observed in the biofilm matrix and were predominantly dead. Alternatively, following the treatment with RP2, the matrix surface appeared like a continuous glycocalyx layer but less homogeneous, and with

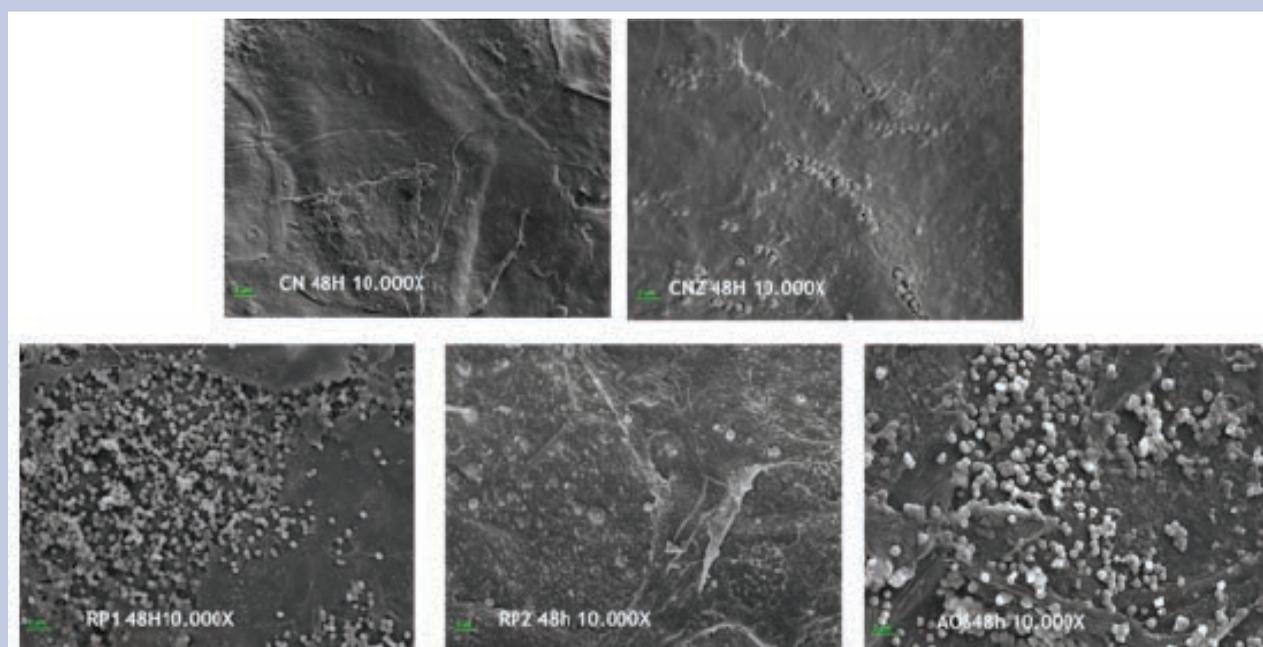


Figure 3. Scanning electron microscopy imaging analysis. Biofilm prevention experiment II. Tissues harvested 4 hours after treatment at 48 hours with reference product (RP) 1, reference product 2, and acid-oxidizing solution, compared with the controls. CN= noncolonized; CNZ= colonized; RP1: reference product 1; RP2: reference product 2; AOS: acid-oxidizing solution

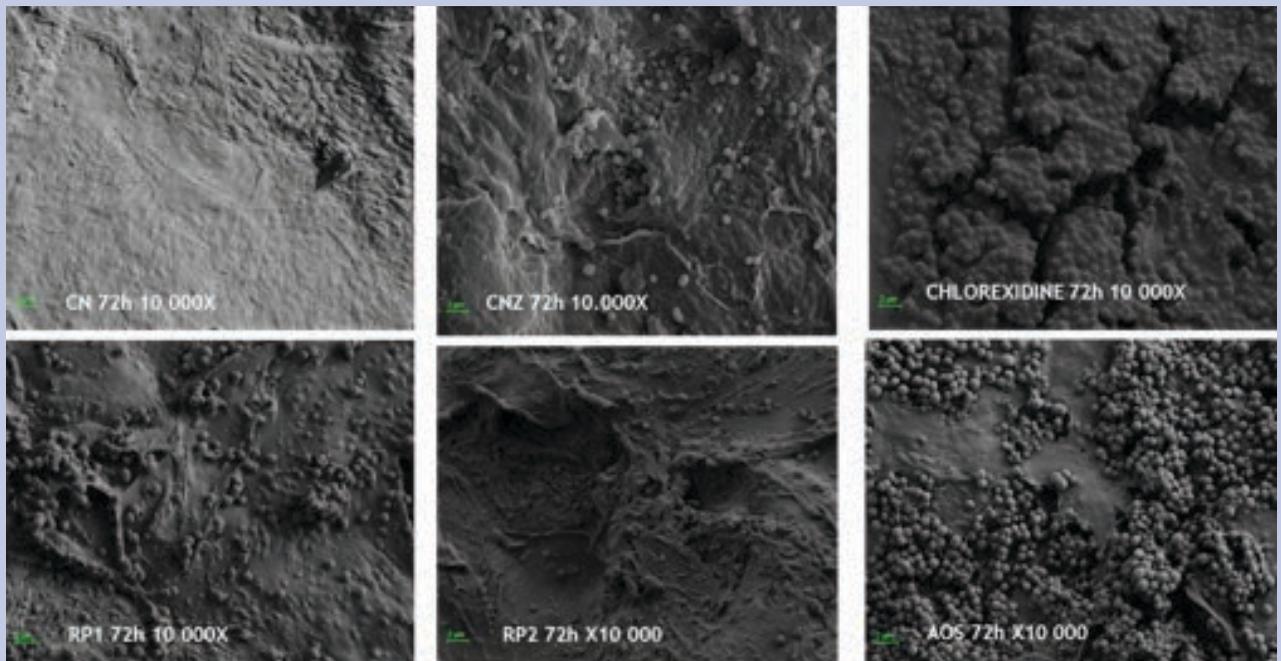


Figure 4. Scanning electron microscopy imaging analysis. Biofilm eradication experiment III. Tissues harvested 4 hours after treatment at 72 hours with reference product (RP) 1, RP2, and AOS compared to controls
CN= noncolonized; CNZ= colonized; RP1: reference product 1; RP2: reference product 2; AOS: acid-oxidizing solution

clear signs of dehydration compared with the biofilm of the control. In the sample treated with AOS, both bacteria and biofilm with mucous structures clearly visible were present. The main difference compared with the colonized control was the predominance of the planktonic phenotype on a modified matrix: single bacteria or microcolonies were visible but appeared free and not included in the glycocalyx (Figure 3).

Biofilm eradication experiment III. An eradication model was set up to evaluate the effect of the study products on an established biofilm. Washings started after 48 hours from the bacterial deposition and were repeated 2 times at 52 hours and 72 hours. The biofilm structure of the colonized untreated control appeared smooth, homogeneous, continuous, and with bacteria entirely embedded. Following treatment with RP1, the epidermal surface was scattered with nonviable colonies and a visible corneocytes structure, suggesting biofilm degradation. However, in the tissue treated with RP2, the epithelium surface was covered by a continuous layer, which hid the corneocytes. Single bacteria or microcolonies were present and appeared well embedded in the EPS matrix. With samples treated with AOS, bacterial colonies were visible on the surface, with bacteria present as separate entities and not

encapsulated in the matrix. Chlorhexidine, the positive control, completely disrupted the biofilm (Figure 4).

Skin compatibility. Acid-oxidizing solution showed good tissue compatibility in comparison with RP1 and RP2. The relative viability was analyzed on noncolonized untreated tissues following scraping and after 4 treatments at 0 hours, 24 hours, 48 hours, and 72 hours with the different test solutions. Samples were harvested 4 hours after treatments at 28 hours, 52 hours, and 76 hours (Figure 5). The results show that, unlike the results from RP1 and RP2, which were characterized by a residual viability of about 50% even after 52 hours, the compatibility of AOS with RHE tissue is much better, resulting in a residual viability higher than 80% at 72 hours.

Discussion

Observations of natural ecosystems led to the conclusion that the majority of microorganisms do not live as free-floating organisms (defined as planktonic bacteria) but in a biofilm, defined as “an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material.”²⁰ Formation of biofilm is an ecological process in which a particular bacterial strain colonizes

Metabolic Activity Assay on Noncolonized Tissues CN = 100

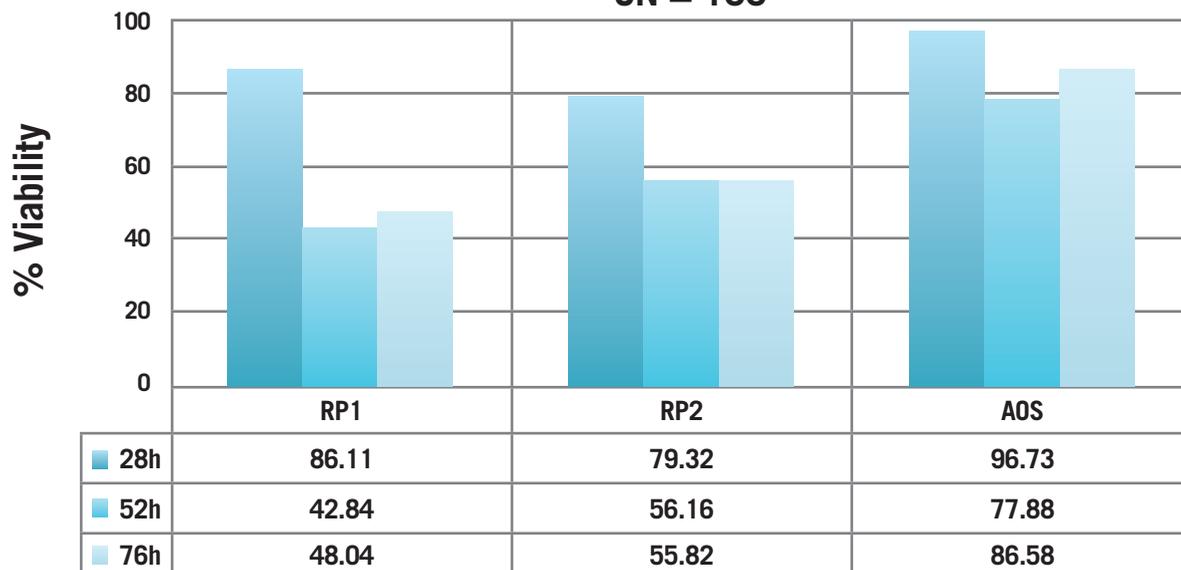


Figure 5. Metabolic activity assay. Tissue residual viability measured after treatments at 0 hours, 24 hours, 48 hours, and 72 hours with samples harvested at 28 hours, 52 hours, and 76 hours. Values were normalized vs noncolonized untreated tissue at each time point (CN = 100% viability) CN: noncolonized; RP1: reference product 1; RP2: reference product 2; AOS: acid-oxidizing solution.

a niche, in spite of adverse environmental factors, that in some cases may lead to the development of a population sufficiently active to exert a pathological effect. Accordingly, in the past few years biofilms deserved high consideration in medical practice since they can highly contribute to the chronicization of wounds favoring persistence of infections, whereas free-floating planktonic bacteria are commonly associated with acute infections. In particular, mature biofilms become extremely resistant to biocidal penetration,²¹ rendering antimicrobials largely ineffective.²²

The *in vitro* evaluation of antibiofilm activity is not an easy task and several models were developed for this purpose, even though some limitations, notably their failure to reproduce the complex host environments, have to be considered when extrapolating results to the *in vivo* situations.²³ In this preclinical study, a model based on 3-dimensional living reconstructed human epidermis was set up in which the epidermal layer was colonized with MRSA and a biofilm was subsequently allowed to form within the following 48 hours. Using this model, the activity of a new acid-oxidizing solu-

tion (AOS), containing free chlorine species of which 95% stabilized hypochlorous acid and characterized by a pH < 3 and super-oxidizing features (> 1000 mV), was investigated on biofilm formation and eradication. The results show that AOS, in 2 different biofilm prevention experiments, was able to induce a morphological change in wound's biofilms model. In fact, at the end of the treatment, AOS was able to clearly increase the presence of the planktonic phenotypes over the encapsulated forms, as observed with SEM showing single bacteria or microcolonies not embedded in the glycocalyx. A similar result was obtained in the biofilm eradication experiment in which, after the treatment of the already formed biofilm with AOS, a partial disruption was observed with bacterial colonies clearly visible as separate entities on the surface of the cell layer but not encapsulated in the biofilm matrix.

In this study, RP1, containing betaine and polyhexanide, affects the biofilm suggesting a disruption effect, as showed by SEM analysis. RP2, a solution with super-oxidizing features, shows, by means of the SEM analysis, to have less power in modifying the biofilm

KEYPOINTS

- The authors hypothesize that acid-oxidizing solution (AOS) may exert a stronger bacterial releasing effect by virtue of the more highly pure content of hypochlorous acid among the free chlorine species associated with a low pH.
- An additional key feature of AOS is a negligible cytotoxicity, a fundamental characteristic in chronic wounds where preservation of cell viability is essential to allow required reconstructive activity of wound repair.
- The presented data show that treatment with AOS is useful not only as preventive application but also when tested in the eradication model.

structure than AOS. The authors hypothesize that AOS may exert a stronger bacterial releasing effect, probably by virtue of the more highly pure content of hypochlorous acid among the free chlorine species associated with a low pH (ie, pH < 3). These data suggest that AOS solution may also be effective in the treatment of chronic wounds where the formation of biofilm is a common event.

An additional key feature of AOS is a negligible cytotoxicity, a fundamental characteristic in chronic wounds where preservation of cell viability is essential to allow required reconstructive activity of wound repair. In such a critical situation, a well-tolerated cleansing solution represents a key element to avoid further delays in wound healing.

Furthermore, the presented data show that treatment with AOS is useful not only as preventive application but also when tested in the eradication model. As a matter of fact, AOS was effective on the formed biofilm structure that became at least partially disrupted, facilitating the release of bacterial colonies trapped in the polymeric matrix. This finding is relevant since Alhede and colleagues²⁴ have shown that higher tolerance towards antibiotics, a typical characteristic of the sessile forms of bacteria, relies on the properties of the matrix itself. Alhede and coauthors²⁴ demonstrated that aggregated cells could be eradicated by a combination of antibiotics and simple mechanical disruption. They concluded that, if disruption procedures could be applied together with high concentrations of locally administered antibiotics, chronic infections might be efficiently cured. Since data from the present study suggest that a similar result could be reasonably obtained with subsequent AOS washings, a much milder

procedure than mechanical disruption, an advantage in patients' acceptance and compliance could also be envisaged.

The most relevant result of this study consists in the demonstration that AOS is able to induce a morphological change in the biofilm of a wound, favoring a partial release of bacteria from the extracellular matrix to become more susceptible to the effects of the endogenous immune response, as well as to the external mechanical and antimicrobial treatments, while preserving cellular safety. In this paper, the authors demonstrated for the first time with a modern and reliable technique—the SEM—the compelling disrupting effect of a hypochlorous acid-containing solution (ie, the AOS) on the structure of the biofilm polymeric matrix, thereby providing evidence for the use of AOS both for the prevention and eradication of wound biofilms.

Conclusion

Acid-oxidizing solution is able to induce morphological changes of biofilms in wounds. These effects were evidenced by SEM in an in vitro model using AOS both to prevent biofilm formation or to eradicate an existing layer of biofilm, resulting in the release of bacteria from the extracellular matrix with a consequent higher bacterial accessibility to the attack of mechanical procedures and of antimicrobial agents. Acid-oxidizing solution also showed a stronger cell viability preservation than RP1 and RP2, suggesting a less-invasive local action. Acid-oxidizing solution can therefore be considered a useful tool in the management of chronic wounds due to its ability to disrupt the structure of the biofilm, thereby assisting with susceptibility to cleansing and ensuring a favorable local tolerability.

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