

Original Article

Reduction of biofilm in chronic wounds by antibacterial protease combined with silver dressing

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Abstract: To evaluate the efficacy of antibacterial protease combined with silver dressing on biofilm reduction and healing in chronic wounds. A pilot, single-blinded randomized controlled trial was conducted. Fifty-six participants with chronic wounds were recruited and randomized (1:1) to the experiment group (antibacterial protease solution + silver dressing, AP) or a control group (0.9% normal saline + silver dressing, NS). The interventions were carried out once two days and the wounds were monitored for 28 days. The wound tissue samples were collected. The bacteria and extracellular polymeric substance in the biofilm of the wound tissues were pathologically checked by transmission electron microscope (TEM) and confocal laser scanning microscope (CLSM). The wound volume reduction rate, Pressure Ulcer Scale for Healing (PUSH) score, pH and temperature were measured. After treatment for 28 days, in the AP group, bacteria and extracellular polymeric substance in the biofilm of the wound tissues decreased more significantly than those in the NS group when observed by CLSM ($P < 0.001$). Furthermore, the AP group displayed more significant reduction in the wound volume reduction rate, Pressure Ulcer Scale for Healing Score (PUSH) and pH compared to the NS group ($P = 0.030, 0.023, 0.015$, respectively). There were no statistical differences in wound temperature between the groups ($P = 0.422$). The application of antibacterial protease combined with silver dressing showed higher efficacy of the biofilm reduction in chronic wounds.

Keywords: Antibacterial protease, biofilm, chronic wounds, silver dressing

Introduction

Chronic wounds are wounds that cannot heal in a timely and effective way according to normal procedures after 4 weeks of clinical treatment, for example, diabetic foot ulcers (DFU), pressure injury, traumatic ulcers, arterial ulcers and venous ulcers [1, 2]. Chronic wounds do not heal within the expected time frame because they remain in the inflammatory phase of the wound-healing process. Previous studies indicated that many chronic persistent infections were the result of the biofilm mode of microbial growth [3, 4]. Within the biofilm, bacteria are cocooned in a self-produced extracellular matrix, which accounts for 90% of the biomass [4]. The matrix comprises extracellular polymeric substance (EPS), which includes exopolysac-

charides, extracellular proteins, extracellular DNA, water, surfactants and lipids, and acts as a stabilizing scaffold to support the three-dimensional biofilm structure [5, 6]. This particular structure protects bacteria in the biofilm from antibacterial agents, the surrounding environment and the host's immune response, increasing their resistance to antibiotics by a factor of 1000 compared with the planktonic status [2]. Biofilms exist widely in chronic wounds, and the incidence has increased annually. As early as 2008, James *et al.* [4] found that 60% of chronic wounds displayed biofilm infection. Recent studies have shown that the incidence of biofilm infection in chronic wounds has reached 60%-100% [2, 4, 7, 8], representing a major challenge for clinical professionals when treating chronic wounds. In addition,

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Khalid *et al.* [8] found that all five acute DFU wounds in his study contained biofilms, an incidence rate of 100%, compared with only 6% for acute wounds (one out of 16 acute wound specimens) in James *et al.* study [4]. The studies of Gurjala and Li *et al.* [9, 10] showed that *Staphylococcus aureus* could rapidly develop and form a mature biofilm within 6-24 hours after inoculation on an acute-wound model of rabbit ears. According to the above findings, it might also be an increasing trend of incidence for biofilm infections in acute wounds. However, there is currently no consensus on the optimal treatment of biofilm-infected wounds.

Silver dressing, broad-spectrum antibacterial dressing is commonly used in clinical practice and has a significant effect on killing bacteria and a certain capacity to resist biofilm *in vitro* [11, 12]. However, silver dressing alone is not ideal in controlling chronic-wound infection or promoting wound healing [13]. Lysozyme has been demonstrated to have a good efficacy against biofilm when tested *in vitro* [14-18]. In the present study, we performed a pilot study combining lysozyme with silver dressing to treat biofilm-infected chronic wounds. The wound tissue samples were collected. The bacteria and extracellular polymeric substance in the biofilm of the wound tissues were pathologically checked by transmission electron microscope (TEM) and confocal laser scanning microscope (CLSM). The wound volume reduction rate, Pressure Ulcer Scale for Healing (PUSH) score, pH and temperature were also measured.

Materials and methods

Participants

During the period from March 2017 to June 2018, 56 patients with chronic wounds were recruited at the Outpatient Wound Care Center of Nanjing General Hospital of Nanjing Military Region. This trial was registered with the International Clinical Trial Registration Platform (ICTPR) via the Chinese Clinical Trial Registry (ChiCTR1800019687).

Inclusion criteria

Wounds that had not healed for more than 4 weeks [1]; in the case of DFU, patients had normal blood glucose during the previous 2 weeks. Wounds that met six clinical characteristics

that may indicate the presence of bacterial biofilm: treatment failure despite using appropriate antibiotics or antiseptics; delayed healing; cycles of recurrent infection/exacerbation; excessive moisture and wound exudate; low-level chronic inflammation; low-level erythema [19]; Age \geq 18 years; voluntarily participation in the clinical research and provision of signed informed consent.

Exclusion criteria

Presence of severe heart, lung, kidney or liver complications or critical illness, life-threatening conditions, including shock; patients with bleeding disorders or bleeding tendency; secondary to systemic infections or other infections requiring systemic antibiotic treatment; pregnant or lactating women; presence of autoimmune disease or taking hormones.

All selected patients signed an informed consent form before starting the study. According to a list of numbers generated by computer software, all patients were randomly assigned to two groups. An independent third-party research group prepared the randomization list and the sealed envelopes. The patients in the AP and NS groups respectively received lysozyme and 0.9% normal saline (NS) solution for the wetting compress on the wound bed as the comparator.

Experimental and control group interventions

This study followed the Consolidated Statement of Reporting Trials (CONSORT). This was a non-blind, two-arm, randomized controlled trial with an intervention group treated with antibacterial protease solution (AP) and control group treated with 0.9% normal saline (NS).

All the selected patients were under the responsibility of a trained wound nurse. The dressing was changed in compliance with the principle of aseptic operation and the moist healing theory. In brief, the wound-care process included the "six steps" of "assessment, cleaning, debridement, wet compressing, dressing filling and packing". The first step is to assess and measure the wound, which is recorded. In the wound assessment process, the area, depth, color, odor, direction and depth of the undermining or tunnel and the amount of exudate were determined and recorded. In the second

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step, the wound bed was cleaned using 0.9% NS from the outside to the inside, while 5-6 cm of skin around the wound was cleaned using 0.5% povidone iodine solution from the inside to the outside. In the third step, different debridement methods were applied as needed. For loose slough, use hemostatic forceps and forceps for scratching; for adherent compact slough or scab, use autolysis debridement or sharp debridement such as scissors and blades. In the fourth step, in the experimental group, antibacterial protease solution (lysozyme 40000 ± 8000 U/ml, lysostaphin: 1.0 ± 0.2 U/ml, pH 5.58, Shanghai Gaoke Biological Engineering Co., Ltd., Shanghai, China, International patent number: PCT/2006/001640, US 8241901) was used to soak the sterile gauze. The soaked gauze was applied to the wound bed (keeping the gauze wet, but without excess solution) and beyond the wound edge for 1-2 cm for 10 minutes. In the control group, 0.9% normal saline was used to wet compress. The rest of the procedure was the same as for the experimental group. The dressings in the fifth step in two groups were both nano silver dressings (Nanjing Jindi Puhui Pharmaceutical Technology Co., Ltd., Nanjing, China), which covered 1-2 cm beyond the wound edge and fully contacted but did not compress the wound bed. In the sixth step, the wounds were wrapped by using sterile gauzes. The dressings were changed every other day. The treatment and observation duration in both groups was 28 days. The treatment was stopped immediately if any adverse reactions occurred, such as allergies.

Wound volume reduction rate

The wound length, width and depth in two groups were determined using the same measuring ruler on day 0 (D0, pre-treatment), day 14 (D14) and day 28 (D28), from which the wound volume was calculated. The wound volume reduction rate = (pre-treatment wound volume - post-treatment wound volume) / (pre-treatment wound volume) \times 100%.

Pressure Ulcer Scale for Healing (PUSH) score

The PUSH score was initially used to quantitatively evaluate the state of pressure ulcers once weekly [20]. This scoring method has since been applied to other types of chronic

wounds [21]. The PUSH score includes three components: wound area, exudate amount and tissue type. When the PUSH score is reduced, treatment is considered effective, whereas when the PUSH score remains constant or is increased, the treatment is considered ineffective. When the PUSH score turned to be "0", it meant that wound has healed [22]. Consequently, the PUSH score was used to measure wound healing status.

pH and temperature

The wound pH and temperature were determined by using the same type of pH paper (Test Paper Factory No. 3, Shanghai, China) and non-contact infrared thermometer (Yuyue Medical Instrument Co. Ltd., Jiangsu, China) immediately after the removal of the wound dressing on D0 (pre-treatment), D14, and D28. The temperature of the wound center and some spots around the wound in directions at 3 o'clock, 6 o'clock, 9 o'clock and 12 o'clock were determined and the mean value was calculated as the final wound temperature [23].

SEM and CLSM

Specimen collection and storage

A 2 mm (width) \times 10 mm (depth) tissue punch biopsy was obtained from the edge of each wound after cleaning the wound with 0.9% NS at every dressing change. Tissue biopsy samples were obtained from all participants at the baseline (D0) and D28. Following the removal, tissue samples were rinsed vigorously in a phosphate-buffered saline (PBS) bath to remove any coagulated blood and reduce the number of planktonic microorganisms. For SEM, tissue specimens were immediately fixed in 2.5% glutaraldehyde (LeiGen biotechnology Co. Ltd., Beijing, China) in a refrigerator at the temperature of 4°C. For CLSM, tissue specimens were embedded in optimal cutting temperature (OCT) compound (Thermo Fisher Scientific, USA) and stored in a refrigerator at the temperature of -8°C

SEM

The wound tissue samples fixed in 2.5% glutaraldehyde were dehydrated by using an increasing ethanol gradient of 50%-100% and dried

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under vacuum for 6 hours. The dried samples were adhered to the double-sided tape on the sample holder, and a gold conductive film was plated on the surface of the sample by vacuum ion sputtering. Finally, the samples were observed and photographed by SEM using a Hitachi 3500 model (Japan). The scanning electron micrograph of each sample was scored based on the amount of bacteria/biofilm observed by using an arbitrary five-point scale as described previously [24]: 0 = no bacteria observed; 1 = single individual cell; 2 = small microcolonies (approximately 10 cells); 3 = large microcolonies (approximately 100 cells); 4 = continuous film; and 5 = thick continuous film.

CLSM

Frozen biopsy tissues embedded in OCT compound were sectioned to a 5 μm thickness and mounted on cationic slides. The slices were rinsed with PBS three times for 5 minutes each and labeled with 5 $\mu\text{g}/\text{ml}$ concanavalin A (Con A, Thermo Fisher Scientific) for 1 hour in the dark at room temperature [25]. Then the slices were rinsed with PBS three times (5 minutes each) and then Hoechst 33342 nucleic acid stain (Thermo Fisher Scientific, USA) was applied for 5 min in the dark at room temperature [26]. Finally, the slices were sealed with anti-bleaching agent. CLSM using a Leica TCS SP8 (Germany) was performed to visualize the fluorescence of each dye. The excitation/emission wavelengths were 405/420-503 nm for the Hoechst 33342 in a blue channel and 560/570-630 nm for con A in a red channel. Five visual fields were randomly selected for each specimen, and the mean value was obtained after using Image J software for fluorescence quantitative analysis (National Institute of Health, USA).

Ethics

This study was approved by the Ethics Committee of Jinglin Hospital, the Affiliated Hospital of Nanjing University Medical School (2014-GJJ-115).

Statistical analysis

Data were analyzed by using a Statistical Package for the Social Sciences (SPSS; IBM Ver-

sion 22.0, 2013, USA) database. All the measurement data are expressed as the mean \pm standard deviation. When the measurement data did not conform to the normal distribution, a T-test analysis was used to compare the differences between the two groups. Otherwise, Mann-Whitney U test was applied for the comparison. Counting data were analyzed by the Chi-square test. The PUSH score, pH and temperature were determined in triplicates and analyzed statistically by repeated analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Results

We recruited 56 patients (28 in each group), while 9 out of the total 65 patients screened were excluded for personal reasons. Furthermore, one patient in the AP group and two in the NS group did not complete the 4-week treatment process because of the change of the treatment methods (the patient of the AP group received a surgery on D21, while the two patients of the NS group underwent negative-pressure wound therapy on D11 and D15 respectively). However, their previous recorded outcomes were included in the overall results assessment to maintain the principle of intention to treat.

Patient characteristics and initial wound assessment

The characteristics of sex, age, body mass index and comorbidities were similar in the two groups. There were six types of chronic wounds contained in each group, including traumatic (32.8%) and arterial ulcers (7.1%) being the most and least frequent wound types respectively. The distribution of wounds by type was also similar in both groups. The comparative analysis did not find any significant difference between the groups. Initially, we recorded the duration of each wound and used professional clinical wound tools to assess the size, depth, pH and temperature of the wound as described above. There were no statistically significant differences between the two groups (**Table 1**).

Reduction of wound volume

In both AP and NS groups, the size and depth of the wounds were determined on D14 and D28. Analysis using T-test for two independent sam-

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Table 1. Patients and wound characteristics at the initial assessment (baseline)

Recruited patients	NS group (n = 28)	AP Group (n = 28)	P-value
Gender			.274*
Male	16 (57.14%)	19 (67.86%)	
Female	12 (42.86%)	9 (32.14%)	
Parameters	Mean ± SD	Mean ± SD	
Age (average)	59.19 ± 13.65	57.30 ± 14.63	.106**
Body mass index (BMI)	22.31 ± 3.79	22.58 ± 3.09	.214**
Wound duration (Days)	82.71 ± 88.04	88.11 ± 73.74	.275**
wound volume (cm ³)	7.67 ± 6.40	8.09 ± 6.83	.523**
PUSH score	11.11 ± 2.50	11.61 ± 2.32	.439***
Temperature (°C)	32.94 ± 1.19	32.66 ± 2.03	.787*
pH	7.29 ± 0.23	7.32 ± 0.19	.193 *
Comorbidities			.716****
None of Hypertension and Diabetes Mellitus	20 (71.43%)	16 (57.14%)	
Hypertension only	4 (14.29%)	5 (17.85%)	
Diabetes Mellitus only	2 (7.14%)	4 (14.29%)	
Both	2 (7.14%)	3 (10.71%)	
Wounds types			.873****
Pressure injury	4 (14.29%)	3 (10.71%)	
DFU	4 (14.29%)	2 (7.14%)	
Venous ulcer	8 (28.57%)	6 (21.43%)	
Arterial ulcer	1 (3.57%)	3 (10.71%)	
Arteriovenous ulcer	2 (7.14%)	5 (17.85%)	
Traumatic ulcers	9 (32.14%)	9 (32.14%)	

*P-value obtained through the χ^2 test; **P-value obtained through the Mann-Whitney U test; ***P-value obtained through the T-test for 2 independent samples; ****P-value obtained through the Fisher's exact test. $P < 0.05$ refers to statistic difference.

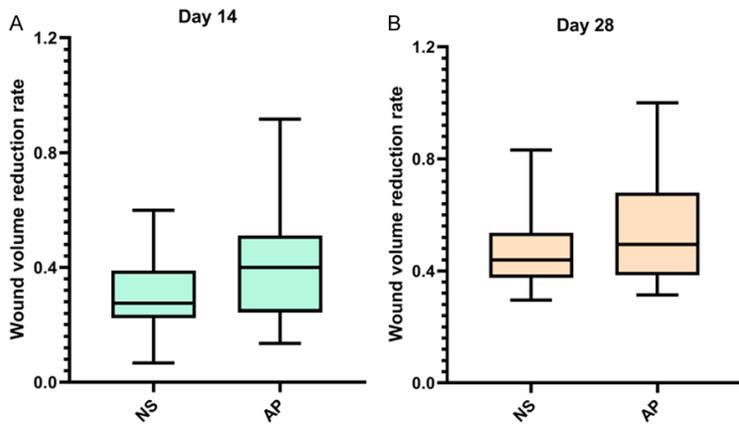


Figure 1. Wound volume reduction rate comparison between the NS and AP groups. (A) Shows the wound volume reduction rate in the NS and AP groups on day 14 and (B) on day 28.

ples showed a statistical difference in the wound volume reduction rate between the two groups on D14 ($31.2\% \pm 11.8\%$ vs $41.7\% \pm 20.2\%$, $P = 0.028$) (**Figure 1A**) and D28 ($46.2\% \pm 12.0\%$ vs $56.5\% \pm 21.1\%$, $P = 0.030$) (**Figure 1B**).

Reduction of biofilm

The reduction of the biofilm was visualized in chronic wounds by SEM and CLSM. The initial median values (D0) of biofilm architecture in the NS and AP groups viewed by SEM were both 5 (thick continuous film). On D28, the median value was reduced to 3 (large microcolonies of approximately 100 cells) in the NS group (**Figure 2A, 2C**) and 2 (small microcolonies of approximately 10 cells) in the AP group (**Figure 2B, 2D**).

By CLSM and Image J software analysis, the reduction in the fluorescence intensity of bacteria between D0 and D28 in the NS vs the AP group was 7.29 ± 1.80 vs 11.97 ± 4.39 ($P < 0.001$) and for the EPS of 9.84 ± 3.30 vs 15.28 ± 5.51 ($P < 0.001$) (**Table 3**). Therefore, the

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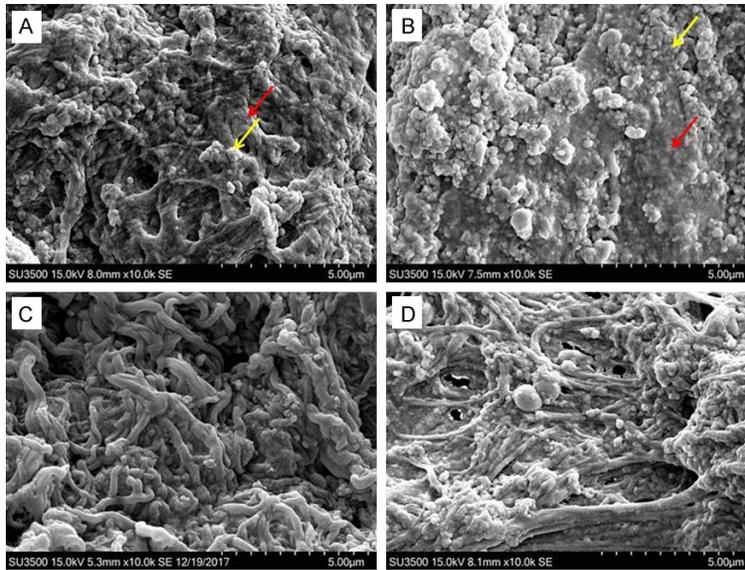


Figure 2. Representative samples observed by scanning electron microscopy. (A) and (B) show large micro-colonies encased in thick extracellular matrix in the NS and AP groups, respectively, on day 0. Yellow arrows indicate bacterial colonies and red arrows represent the extracellular polymeric substance (EPS) that surrounds the bacteria (biofilm score: 5). (C) Shows large microcolonies without EPS or fibrous tissue (biofilm score: 3). (D) Shows small microcolonies without EPS but fibrous tissue exists (biofilm score: 2).

reduction of bacteria and EPS were apparently greater in the AP group (Figure 3A-C, 3a-c) than the NS group (Figure 3D-F, 3d-f) after 28 days of treatment.

PUSH score, temperature and pH

Repeated ANOVA was applied to compare the differences among PUSH score, temperature and pH by using SPSS 22.0 software. For the PUSH score, there were statistical differences in time factor, the interaction between time factor and group factor, group factor. This result illustrated that PUSH score had a tendency to change with time, the effect of time factor varied with grouping and there was a statistically significant difference in PUSH score ($P_{\text{time}} < 0.001$, $P_{\text{time+group}} < 0.001$, $P_{\text{group}} = 0.023$) between these two groups. Lysozyme effectively reduced the wound pH ($P_{\text{time}} < 0.001$, $P_{\text{time+group}} < 0.001$, $P_{\text{group}} = 0.015$). However, there was no significant difference in temperature between the two groups ($P_{\text{time}} = 0.833$, $P_{\text{time+group}} = 0.183$, $P_{\text{group}} = 0.422$) (Table 2).

Discussion

By using a combination of clinical assessment methods, SEM and CLSM, the universal presence of a biofilm in chronic wounds has been

investigated. To the best of our knowledge, this is the first time that the combination of clinical measurements and microscopy techniques have been applied to show that lysozyme combined with silver dressing can reduce the biofilm and, therefore, accelerate chronic-wound healing. In contrast to silver dressing alone in the NS group, AP group, which additionally included lysozyme solution clearly reduced bacteria and EPS in the biofilm as observed by SEM and CLSM (Figures 2 and 3). Furthermore, the wound volume of the AP group was reduced more significantly compared with the NS group. These results were similar to previous *in vitro* studies. Ceotto-Vigoder *et al.* [16] confirmed that lysostaphin treatment ($0.4 \mu\text{g ml}^{-1}$) for 4 h induced strong *S. aureus* bio-

film detachment and the death of most of the sessile cells *in vitro*. When Wu *et al.* first demonstrated lysostaphin activity against staphylococcal biofilms, they proposed that the mechanisms of biofilm elimination by lysostaphin might include the rapid lysis of adherent cells among others, which may be sufficient to destabilize biofilm matrix and allow the detachment of the adherent cells from the matrix [27]. A series of *in vitro* experiments similarly confirmed the inhibitory effect of lysozyme on different forms of bacterial biofilms. For example, Hou *et al.* [17] combined lysozyme and liposomal gentamicin (LLG) for the treatment of biofilms, which indicated that LLG was more effective at disrupting established biofilms and inhibiting biofilm formation by pathogens, including gram-positive and gram-negative bacteria, than gentamicin alone. Therefore, lysozyme was also able to act on biofilms. In our study, it could be suggested that lysostaphin and lysozyme as the constituents of the antibacterial protease used in the experimental group probably reduce biofilm synergistically.

According to the data above, we first hypothesized that the EPS of the biofilm was broken down by lysozyme. Subsequently, silver dressing killed the plankton bacteria released from the biofilm. Finally, removal of the bacterial bio-

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Table 2. Wound PUSH score, temperature and pH after treatment

	Group	D0	D14	D28	F_{time}	$F_{\text{time+group}}$	F_{group}
PUSH score	NS	11.11 ± 2.50	9.00 ± 2.49	6.14 ± 2.19	243.85	22.69	5.46
	AP	11.61 ± 2.32	6.57 ± 2.06	4.04 ± 1.97			
P-value					< 0.001	< 0.001	.023
temperature	NS	32.94 ± 1.20	33.22 ± 1.12	32.81 ± 1.40	.18	1.73	.65
	AP	32.66 ± 2.03	32.61 ± 1.12	33.03 ± 1.46			
P-value					.833	.183	.422
pH	NS	7.29 ± 0.23	7.30 ± 0.20	7.26 ± 0.22	16.74	9.00	6.35
	AP	7.32 ± 0.19	7.20 ± 0.09	7.04 ± 0.14			
P-value					< 0.001	< 0.001	.015

$P < 0.05$ refers to statistic difference.

Table 3. Fluorescence quantitative analysis results comparing between D0 and D28

Group	Blue staining			Red staining		
	D0	D28	Difference value	D0	D28	Difference value
NS	22.10 ± 7.33	14.88 ± 6.76	7.29 ± 1.80	34.28 ± 9.38	24.52 ± 10.48	9.84 ± 3.30
AP	23.20 ± 3.96	11.23 ± 4.70	11.97 ± 4.39	33.87 ± 7.10	18.85 ± 7.35	15.28 ± 5.51
P-value			< 0.001**			< 0.001**

** P -value obtained through the Mann-Whitney U test; $P < 0.001$ refers to significant statistic difference.

film was achieved and there was no opportunity for the plankton bacteria to reestablish the biofilm.

In the present study, there was no difference in temperature but a significant difference in pH after intervention for 28 days between the two groups (Table 3). Our data suggest that improved outcome of the chronic wounds of the AP group treated by lysozyme and the silver dressing may be associated with the pH, an important factor of the wound microenvironment [28]. The pH of the antibacterial protease solution in our study was 5.58. The Skin is physiologically acid with a normal pH range of 4.8-6, mainly resulting from the organic acid secretion by keratinocytes [29]. The normally acidic surface pH helps protect the organism from bacteria and fungi that require a pH > 6 to thrive [30]. When disruption of the skin integrity occurs, the pH of the wound's surface tends to increase because of the diffusion of more alkaline interstitial fluids and of plasma extravasation from injured capillaries [29]. Furthermore, biofilm formation causes an increase in pH near the tissue surface, thereby delaying the wound healing [31, 32]. A low pH environment could increase oxygen release from the blood compartment, which is particularly significant for chronic wounds, because hypoxia repre-

sents one of the most important factors that impair the healing process [28, 33]. Additionally, an acidic microenvironment is thought to reduce the risk of infections and facilitate granulation tissue formation [34, 35]. By contrast, an alkaline microenvironment impairs wound healing by allowing bacterial infections and biofilm formation [36].

Conclusion

In conclusion, antibacterial protease combined with silver dressing can reduce biofilm formation and promote wound repair effectively. Based on more clinical and experimental research, this combined treatment could become a potential anti-biofilm therapeutic strategy for chronic wounds in the future.

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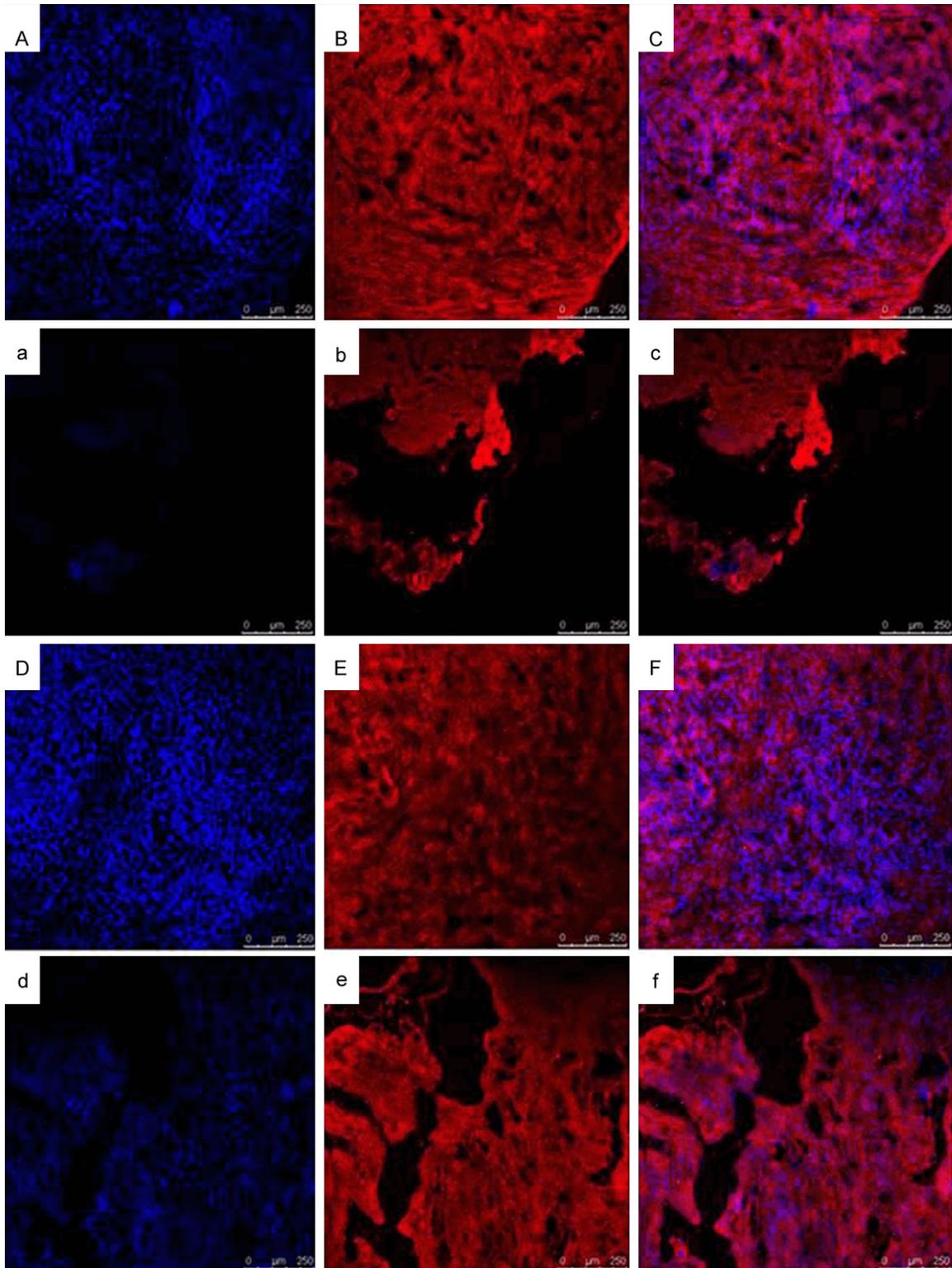


Figure 3. Representative samples observed by confocal laser scanning microscopy. Concanavalin A lectin was used to stain exopolysaccharides (red) of EPS and Hoechst 33342 was applied to stain the bacterial nucleic acids (blue). (A-C) and (a-c) are observations on days 0 and 28 in the AP group; (D-F) and (d-f) are observations on days 0 and 28 in the NS group.

Disclosure of conflict of interest

None.

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References

- [1] Frykberg RG and Banks J. Challenges in the treatment of chronic wounds. *Adv Wound Care (New Rochelle)* 2015; 4: 560-582.
- [2] Nunan R, Harding KG and Martin P. Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. *Dis Model Mech* 2014; 7: 1205-1213.
- [3] Kucisec-Tepes N. The role of antiseptics and strategy of biofilm removal in chronic wound. *Acta Med Croatica* 2016; 70: 33-42.
- [4] James GA, Swogger E, Wolcott R, Pulcini E, Seccor P, Sestrich J, Costerton JW and Stewart PS. Biofilms in chronic wounds. *Wound Repair Regen* 2008; 16: 37-44.
- [5] Kostakioti M, Hadjifrangiskou M and Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med* 2013; 3: a010306.
- [6] Alavi MR, Stojadinovic A and Izadjoo MJ. An overview of biofilm and its detection in clinical samples. *J Wound Care* 2012; 21: 376-383.
- [7] Hurlow J, Blanz E and Gaddy JA. Clinical investigation of biofilm in non-healing wounds by high resolution microscopy techniques. *J Wound Care* 2016; 25: S11-S22.
- [8] Johani K, Malone M, Jensen S, Gosbell I, Dickson H, Hu H and Vickery K. Microscopy visualisation confirms multi-species biofilms are ubiquitous in diabetic foot ulcers. *Int Wound J* 2017; 14: 1160-1169.
- [9] Gurjala AN, Geringer MR, Seth AK, Hong SJ, Smeltzer MS, Galiano RD, Leung KP and Mustoe TA. Development of a novel, highly quantitative in vivo model for the study of biofilm-impaired cutaneous wound healing. *Wound Repair Regen* 2011; 19: 400-410.
- [10] Li T, Zhang L, Han LI, Wang G, Yin P, Li Z, Zhang L, Guo QI, Liu D and Tang P. Early application of negative pressure wound therapy to acute wounds contaminated with *Staphylococcus aureus*: an effective approach to preventing biofilm formation. *Exp Ther Med* 2016; 11: 769-776.
- [11] Boateng J and Catanzano O. Advanced therapeutic dressings for effective wound. *J Pharm Sci* 2015; 104: 3653-3680.
- [12] Warriner R and Burrell R. Infection and the chronic wound: a focus on silver. *Adv Skin Wound Care* 2005; 18: 2-12.
- [13] (NICE). NifHaCE. Chronic wounds: advanced wound dressings and antimicrobial dressings [EB/OL]. nice.org.uk/guidance/esmpb2, 20-16.
- [14] Walencka E, Sadowska B, Rozalska S, Hryniewicz W and Rozalska B. Lysostaphin as a potential therapeutic agent for staphylococcal biofilm eradication. *Pol J Microbiol* 2005; 54: 191-200.
- [15] Aguinaga A, Frances ML, Del Pozo JL, Alonso M, Serrera A, Lasa I and Leiva J. Lysostaphin and clarithromycin: a promising combination for the eradication of *Staphylococcus aureus* biofilms. *Int J Antimicrob Agents* 2011; 37: 585-587.
- [16] Ceotto-Vigoder H, Marques SL, Santos IN, Alves MD, Barrias ES, Potter A, Alviano DS and Bastos MC. Nisin and lysostaphin activity against preformed biofilm of *Staphylococcus aureus* involved in bovine mastitis. *J Appl Microbiol* 2016; 121: 101-114.
- [17] Hou Y, Wang Z, Zhang P, Bai H, Sun Y, Duan J and Mu H. Lysozyme associated liposomal gentamicin inhibits bacterial biofilm. *Int J Mol Sci* 2017; 18.
- [18] Ely VL, Vargas AC, Costa MM, Oliveira HP, Pötter L, Reghelin MA, Fernandes AW, Pereira DIB, Sangioni LA and Botton SA. *Moraxella bovis*, *Moraxella ovis* and *Moraxella bovoculi*: biofilm formation and lysozyme activity. *J Appl Microbiol* 2019; 126: 369-376.
- [19] Schultz G, Bjarnsholt T, James GA, Leaper DJ, McBain AJ, Malone M, Stoodley P, Swanson T, Tachi M, Wolcott RD; Global Wound Biofilm Expert Panel. Consensus guidelines for the identification and treatment of biofilms in chronic nonhealing wounds. *Wound Repair Regen* 2017; 25: 744-757.
- [20] Thomas DR, Rodeheaver GT, Bartolucci AA, Franz RA, Sussman C, Ferrell BA, Cuddigan J, Stotts NA and Maklebust J. Pressure ulcer scale for healing: derivation and validation of the PUSH tool. The PUSH Task Force. *Adv Wound Care* 1997; 96-101.
- [21] Jiang QX, Wang GL and Weng ZQ. Effect evaluation of different classification wounds with pressure ulcer scale for healing. *J Med Postgrad* 2017; 30: 436-439.

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- [22] Hon J, Lagden K, McLaren AM, O'Sullivan D, Orr L, Houghton PE and Woodbury MG. A Prospective multicenter study to validate use of the PUSH in patients with diabetic, venous, and pressure ulcers. *Ostomy Wound Manage* 2010; 56: 26-36.
- [23] Jiang QX, Zhu LX, Li XH, Peng Q and Zhou X. Effects of wound bed temperature and pH in chronic wounds treated by negative pressure wound therapy with two types of filling dressings. *Nurs J Chin People Liberation Army* 2013; 30: 3-6.
- [24] Han A, Zenilman JM, Melendez JH, Shirliff ME, Agostinho A, James G, Stewart PS, Mongodin EF, Rao D, Rickard AH and Lazarus GS. The importance of a multifaceted approach to characterizing the microbial flora of chronic wounds. *Wound Repair Regen* 2011; 19: 532-541.
- [25] Maeyama R, Mizunoe Y, Anderson JM, Tanaka M and Matsuda T. Confocal imaging of biofilm formation process using fluoroprobed *Escherichia coli* and fluoro-stained exopolysaccharide. *J Biomed Mater Res A* 2004; 70: 274-282.
- [26] Bales PM, Renke EM, May SL, Shen Y and Nelson DC. Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens. *PLoS One* 2013; 8: e67950.
- [27] Wu JA, Kusuma C, Mond JJ and Kokai-Kun JF. Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. *Antimicrob Agents Chemother* 2003; 47: 3407-14.
- [28] Kruse CR, Nuutila K, Lee CC, Kiwanuka E, Singh M, Caterson EJ, Eriksson E and Sorensen JA. The external microenvironment of healing skin wounds. *Wound Repair Regen* 2015; 23: 456-64.
- [29] Scalise A, Bianchi A, Tartaglione C, Bolletta E, Pierangeli M, Torresetti M, Marazzi M and Di Benedetto G. Microenvironment and microbiology of skin wounds: the role of bacterial biofilms and related factors. *Semin Vasc Surg* 2015; 28: 151-159.
- [30] O'Meara SM, Cullum NA, Majid M and Sheldon TA. Systematic review of antimicrobial agents used for chronic wounds. *Br J Surg* 2001; 88: 4-21.
- [31] Lone AG, Atci E, Renslow R, Beyenal H, Noh S, Fransson B, Abu-Lail N, Park JJ, Gang DR and Call DR. *Staphylococcus aureus* induces hypoxia and cellular damage in porcine dermal explants. *Infect Immun* 2015; 83: 2531-2541.
- [32] Guo S and Dipietro LA. Factors affecting wound healing. *J Dent Res* 2010; 89: 219-229.
- [33] Kiamco MM, Atci E, Mohamed A, Call DR and Beyenal H. Hyperosmotic agents and antibiotics affect dissolved oxygen and pH concentration gradients in *Staphylococcus aureus* biofilms. *Appl Environ Microbiol* 2017; 83: e02783-02716.
- [34] Gethin G. The significance of surface pH in chronic wounds. *Wounds UK* 2007; 3: 52-56.
- [35] Hunt TK, Hopf H and Hussain Z. Physiology of wound healing. *Adv Skin Wound Care* 2000; 13: 6-11.
- [36] Stewart CM, Cole MB, Legan JD, Slade L, Vandeven MH and Schaffner DW. *Staphylococcus aureus* growth boundaries: moving towards mechanistic predictive models based on solute-specific effects. *Appl Environ Microbiol* 2002; 68: 1864-1871.