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ORIGINAL ARTICLE

In vivo efficiency of the produced recombinant lysostaphin antimicrobial peptide in treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) skin infection in a mouse model

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ABSTRACT

Background and Objectives: *Staphylococcus simulans* secretes an antimicrobial compound called lysostaphin, which has bactericidal properties. It destroys staphylococci through the hydrolysis of peptidoglycan in the cell wall. Therefore, this unique property indicates the high ability of lysostaphin in the treatment of staphylococcal infections and is considered as an anti-staphylococcal agent.

Materials and Methods: *Escherichia coli* BL21 (DE3) competent cells were transformed with pET32a-lysostaphin clone and induced by isopropyl-β-D-thio-galactoside (IPTG). The recombinant protein was purified by affinity chromatography. Recombinant lysostaphin -A-based ointment was used for external wound healing in animal model. *In vivo* activity of ointment was evaluated by clinical evidences and cytological microscopic assessment.

Results: Our results showed the recombinant protein was produced exactly. The results of checkerboard tests showed MIC, MBC and antibacterial activity test an acute reduction of cell viability during the use of lysostaphin, and SEM results approved the intense wrecking effects of lysostaphin in combination on bacterial cells. Macroscopic findings and microscopic data showed that the recombinant lysostaphin ointment was effective on excisional wound healing.

Conclusion: Our findings proved that the recombinant lysostaphin ointment was effective on wound healing due to *Staphylococcus aureus* infection.

Keywords: Anti-bacterial agents; Lysostaphin; *Staphylococcus aureus*; Wound healing

INTRODUCTION

Infectious diseases are one of the causes of high mortality occurring around the world annually (1). Despite many advances made in the treatment of such diseases, they are still at a high level of danger. In recent years, along with the medical progress, the risk of infectious diseases has increased in patients having special conditions including organ transplants, oncology treatments, intensive care units, and complex surgeries. The infection in burn patients is highly important, since burn wounds disrupt skin integrity and are very susceptible to infection, so that burn wound infections are the cause of death in 60% of burn patients and 300,000 deaths worldwide annually (2). Among the Gram-positive bacteria, methicillin resistant *S. aureus* (MRSA) is a predominant pathogen in nosocomial, community-acquired, and burn wound infections. Colonization is the first stage in the pathogenesis of *S. aureus* infection. Asymp-

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tomatic colonized individuals are a source of the human-to-human transmission of this disease (3). After colonization and invasion of tissue by creating an abscess, this bacterium can cross the host immune system and enter the bloodstream (4). The bacterium also causes diseases in the hosts by producing enterotoxins acting as super antigenes (SAgs). Finally, activation of the variable region of the sequence Vβ in the reception of T (TCR) leads to autoimmune-like responses, which could in turn be life threatening. Therefore, due to high risks and high pathogenicity as well as antibiotic resistance in MRSA, it is necessary to develop new and active therapeutic agents against this group of pathogens (5). *S. simulans* secretes an antimicrobial compound called lysostaphin, which has bactericidal properties. It destroys staphylococci through the hydrolysis of peptidoglycan in the cell wall (6, 7). This unique property of lysostaphin indicates its high potential to treat staphylococcal infections, so that it has been considered an anti-staphylococcal agent (8). *In vivo* and *in vitro* studies on lysostaphin have also indicated that it has the potential to be used alone or in combination with other antibacterial agents to prevent and treat various infectious diseases caused by staphylococci (9).

Moreover, development of the recombinant protein in medicine has led to manufacturing products that directly or indirectly affect the improvement of human health as well as public health (10). Therefore, in the present study, our aim was to investigate in vivo efficiency of the produced recombinant lysostaphin antimicrobial peptide in treatment of MRSA skin infection in a mouse model.

MATERIALS AND METHODS

Ethical approval. This study was approved by the ethics committee of Arak University of Medical Sciences, Arak, Iran, Ethics code: 91-128-6.

Materials and chemicals. Ampicillin, bacterial culture mediums and commercial lysostaphin were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Ni-NTA kit was purchased from QIAGEN Co (USA). Ketamine was purchased from Rotexmedica. Germany and xylazine from Alfasan, the Netherlands.

Gene, vector and bacterial strains. pET32a plasmid with lysostaphin gene (pET-lys) was obtained from an project done by Farhangnia (11). The antibacterial activity and MIC test were assessed against Methicillin-resistant *S. aureus* (MRSA) (ATCC 25923) microorganism. *Escherichia coli (E. coli)* BL21 (DE3) pLysS (Novagen, USA) was used as the recombinant protein expression host.

Expression of lysostaphin in *E. coli* BL21 (DE3) pLysS. One of the recombinant colonies grown on plates containing ampicillin + chloramphenicol was incubated in 2 mL NB medium containing the same two antibiotics at 37ºC. Then, 300 µL of overnight culture was inserted in 50 mL NB broth containing 100 mg/ mL ampicillin+100 mg/ mL chloramphenicol and incubated at 37ºC at 220 rpm. The cells optical density at 600 nm was measured occasionally, and when the culture turbidity was equal to 0.6, isopropyl thio-D-galactosidase (IPTG) (1 mM) was added to induce protein expression. Four hours after induction, whole induced transformed bacteria were harvested by centrifugation at 5000 rpm for 20 min and the pellets were stored at -20ºC.

Protein was analysed by SDS-polyacrylamide gel electrophoresis [SDS-PAGE (12%)] and then gel was stained with komasiblue (11).

Purification of lysostaphin. The bacterial deposition was denaturing with 8M urea and expressed protein was purified by Ni-NTA agarose resin. Protein was analyzed by SDS-polyacrylamide gelelectrophoresis [SDS-PAGE (12%)] (12).

Urea assay test. To ensure the absence of urea in the protein solution, urea assay test was done with kit Berthelot (IRAN) according to the kit protocol.

Molecular dynamics simulation and refolding optimization of lysostaphin. Molecular dynamics simulation was estimated by ExPASy server, Aggrescan Server, Protein Data Bank, PubChem site, HyperChem, chimera, AutoDock, and LigPlot software according to project done by Abbasian (13).

In protein purification urea (8 M) was used, which caused the correct folding of the protein to collide. So, the purified protein was refolded by dialysis process in PBS buffer 20mM with proline 150Mm, glucose 200Mm, pH 8, 4ºC for 24 h according to project done by Abbasian (13).

Then, refolded protein was analyzed by SDS-polyacrylamide gelelectrophoresis [SDS-PAGE (12%)].

The quantity of protein was obtained with the assessment of the ratio of absorbance at 280 and 260 nm and then calculated from the following equation:

(1.55 × OD280) – (0.76 × OD260) = The amount of protein (mg/ mL) (13).

At the same time, the concentrated protein was qualitatively examined by vertical electrophoresis.

***In vitro* tests: preparation of the commercial lysostaphin solution.** Sterile distilled water was used for solvent of commercial lysostaphin. The primary concentration of commercial lysostaphin in solution was 5 mg/mL.

Determination of antibacterial minimum inhibitory concentration (MIC). First, we cultured the bacteria onto agar plates without inhibitor, and the plates were incubated for 18-24 hours at 37°C. For each isolate, three to five morphologically similar colonies were selected from agar plates and transferred into a 5 mL glass tube MH-Broth, and were incubated at 37°C. Regular OD450 was prepared from bacterial culture, and dilutions was made in 2 mL MHB to OD450 = 0.01 then MIC test was performed on MRSA (ATCC 25923) and was done according to CLSI protocol MO7-A10 (14).

In this test the highest proteins concentration was related to the first well and the protein concentration in the next wells was halved, respectively (Well number 1-10). The proteins concentration was 0.1 mg/ mL in first well. Positive control and negative control were MHB (Well number 12) and culture of MRSA (Well number 11) respectively. In addition, MIC experiments were performed to evaluate the effect of commercial lysostafin at the same concentration on the above-mentioned bacteria. To exclude any errors, MIC test was repeated three times. To view the test results of MIC was used resazurin. The blue color indicated the reduced number or absence of bacteria. The red color also indicates the growth of the bacteria.

Determination of minimum bactericidal concentration (MBC). After MIC test, 100 µL microplate blue wells corresponding to the MIC, and the above MIC values of recombinant lysostaphin and commercial lysostaphin were cultured on BHI agar. Then, plates were incubated in 37ºC, 24 h to indicate the lowest lysostaphin and commercial lysostaphin concentrations that led to no colony growth, which was considered as the MBC value. The antibacterial activities were defined according to project done by Fahimirad (15).

Antibacterial activity test. The *in vitro* antibacterial activity of the lysostaphin against MRSA was investigated with the turbidimetric assay. Lysostaphin activity was calculated by the cell lysis monitoring of MRSA cell suspension. The amount of cell lysis is directly related to the reduction of the bacterial cell suspension optical density (OD) at 600 nm.

After that, 200 µL of bacterial cultures was allocated to separate culture tubes and 2× MIC concentrations were added to particular tubes. Tubes were incubated at 37°C. At specific intervals, turbidity was measured at 600 nm. Antibacterial activity test was done according to project done by Abbasian (13). Positive control samples were commercial lysostaphin by concentration of 25 µg/ mL, control samples were recombinant lysostaphin by concentration of 25 µg/ mL and negative control samples were tube without of lysostaphin. The assay was carried out in triplicate.

SEM microscopy. To determine the effect of recombinant lysostaphin on MRSA cells, we used SEM microscopy assay. Preparation of samples was done according to project done by Abbasian (13). The changes in cell morphology were determined by SEM microscopy (AIS2100, Seron Technologies, Uiwang-si, Gyeonggi-do, Korea).

***In vivo* tests: animal model.** This study was approved by the internal animal ethics committee of the Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, IR Iran in accordance with Portuguese law. The Syrian mice were adult males. The mice weighed about 20-25 grams and were obtained from the Pasteur Institute. Animals kept in suitable animal room with temperature 20-25°C, humidity 50%-70% and a 12:12 hr. light/ dark cycle. Rats were nourished by specific rodent pellet and urban tap water.

Culture of MRSA. One colony of MRSA was grown in BHI broth then the bacteria were centrifuged at 10 000 ×g for 10 min, washed, and resuspended in sterile phosphate-buffered saline (PBS). This process was repeated twice, and the bacterial suspension was adjusted to a final density of 1.5×10^8 colony-forming units CFU/mL.

Creating an infectious wound in rats. In this study, 21 rats were divided into 3 groups equally:

A group: Control group that were treated with recombinant lysostaphin-based ointment: lysostaphin /

userine: 100 µg/100 w/w.

B group: Negative control group that were treated with just userine.

C group: Possitive control group that were treated with commercial lysostaphin-based ointment: lysostaphin / userine : 100 µg/100 w/w.

Rats were anaesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine. The panniculus carnosus muscle was selected and area was shaved for 25 mm×20 mm (Fig. 1). Wound was not sutured or covered (k15). Cell suspension provided (10 µl) was equally inoculated on the wound of the mice. All animals were colonized by 1.5× 10^8 CFU of MRSA.

Fig. 1. Creates wound on the skin of the rat on the 0^{th} day

Checking of infectious wound in rats. For this purpose, wound sampling of mice was done 2 days after infection and was culture on blood agar medium.

Drug treatment. After making sure the mice are infected, groups A and C were treated with recombinant lysostaphin and commercial lysostaphin based ointment locally and cutaneously at a dose of 100 µg/100 w/w. The ointment was applied to each mouse twice a day. Group B was treated with userine. Treatment was continued for 7 days (14 times with a 12 hour interval) (16). The wounds were measured on a daily basis using a ruler to assess the wound healing (17).

The percentage of wound healed postsurgical was calculated as follows:

Wound healing percentage= ((A0-Ascar)/A0) ×100% (17).

Where A0 and Ascar are original wound and scared areas, respectively.

Tissue extraction and morphometric examination. For microscopic analysis, on day 7, rats were anesthetized by high inhalation dose of ether. The complete area of the wound including the incision area and adjacent normal skin was came out. Excised tissues were fixed in 10% formalin pH 6.8 and embedded in paraffin. Sectioning and staining was conducted according to H&E protocol staining and finally 7 µm sections were prepared. Assessment was performed by light microscope and for this purpose we used a 10 × 40 objective lens (NIKON Eclipse E2000-Videocamera DS-Fi1, Japan) (18).

RESULTS

Expression and purification of lysostaphin. Protein production was analyzed with SDS-PAGE. The protein produced after 4 h of induction is indicated by the corresponding arrows in Fig. 2A.

SDS-PAGE was used to analyze the quality of purified proteins before and after refolding. The molecular weight of recombinant lysostaphin is 27 kDa, because additional peptide sequences were added by the pET-32a vector, about 20 kDa were added to the size of recombinant lysostaphin, and its molecular weight was observed on the gel to be 47 kDa (Fig. 2B).

Urea assay test. This test is important owing to the presence of urea since it has antibacterial properties and can interfere with the results of *in vitro* and *in vivo* tests. The test method was performed according to the kit instructions.

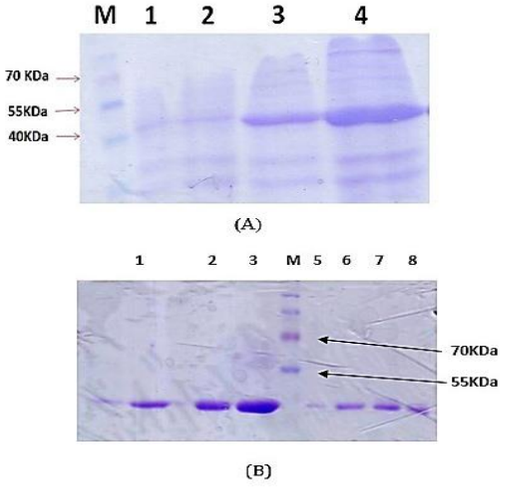

Fig. 2. SDS-PAGE analysis of Lysostaphin in *E. coli* BL21 (DE3) pLysS and purification and refolding of recombinant Lysostaphin. A: Lane M: Protein marker; Lane 1 and 2: pET32a-lys before induction; Lanes 3: pET32a-lys after 2 h; Lane 4: pET32a-lys after 4h. B. Lane 1, 2, 3: purification of recombinant Lysostaphin.; lane M: Protein marker lane 5, 6, 7, 8: protein after refolding.

Molecular dynamics simulation and refolding of lysostaphin. AutoDock software based on the electrostatic force and shape complimentarily was used for molecular dynamics simulation, Fig. 3. According to the results of molecular docking, the amino acid proline had the greatest effect on protein refolding. Lysostaphin concentration after the dialysis procedure was about 0.2 mg/ mL.

***In vitro* tests: determination of MIC.** Our result showed that MIC values of recombinant lysostaphin and comercial lysostaphin against MRSA were 6 µg/ mL and 1.5 µg/ mL respectively (Table 1).

Determination of MBC. The results of the minimum bactericidal concentration (MBC) studies are shown in Table 1. The calculated minimum bactericidal concentration (MBC) / MIC values determined the bacteriostatic and bactericidal activity of recombinant lysostaphin and comercial lysostaphin against MRSA.

Antibacterial activity curves. Rate of lysis of cell has a direct correlation with the loss of optical density of MRSA bacterial suspension in OD600 nm. Our results showed that recombinant lysostaphin lysed the bacterial suspension more than commercial lysostaphin at the same time, and the amount of MRSA in the negative sample increased with time. The results of turbidumetric for negative sample, positive sample and control sample are shown in Fig. 4.

SEM microscopy. To observe the effect of recombinant lysostaphin against MRSA, an electron microscope was used to show the antibacterial effect of recombinant lysostaphin, which can kill bacteria by making holes in their membranes. These results are shown in Fig. 5.

***In vivo* tests.** For checking infectious wound in rats, wound sampling of mice was done 2 days after infection and cultured in blood agar medium. Growth of MRSA on blood agar culture medium showed that high level MRSA was colonized in rat skin.

Fig. 6 shows the results of wound healing on days 4 and 7 after infection. Our results showed a faster wound closure rate in group A compared to B and C groups.

Also, calculation of wound healing percentage at defined days after treatment revealed that group A caused the most increased healing percentage compared to other groups (groups A= 87.75 %, groups B= 57.75%, groups C=75%) (Fig. 7).

Results of microscopic assessment approved that granulation tissue formation and its tissue expanding in A group was less than in B and C groups. Therefore, tissue merge of lesion in A group was more than other groups (Fig. 8).

Fig. 3. Molecular docking results

Table 1. The minimum bactericidal concentrations of recombinant lysostaphin and comercial lysostaphin against MRSA.

	Recombinant Lysostaphin	**Commercial Lysostaphin**
Microorganism	MRSA	MRSA
MIC MBC	6 µg/ mL	1.5 µg/ mL
MBC/MIC	6 µg/ mL	1.5 µg/ mL
Interpretation	1 Bactericidal	1 Bactericidal

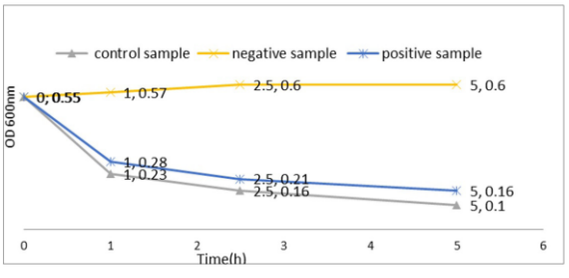

Fig. 4. Antibacterial activity of lysostaphin against MRSA.

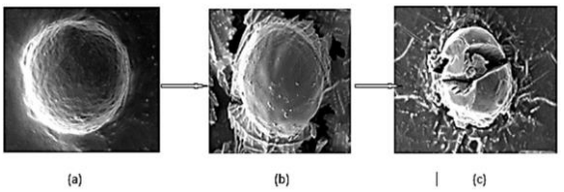

Fig. 5. Therapeutic effects of lysostaphin in the mouse challenge model: (a) SEM micrographs of untreated MRSA (b, c) MRSA cell treated with recombinant lysostaphin.

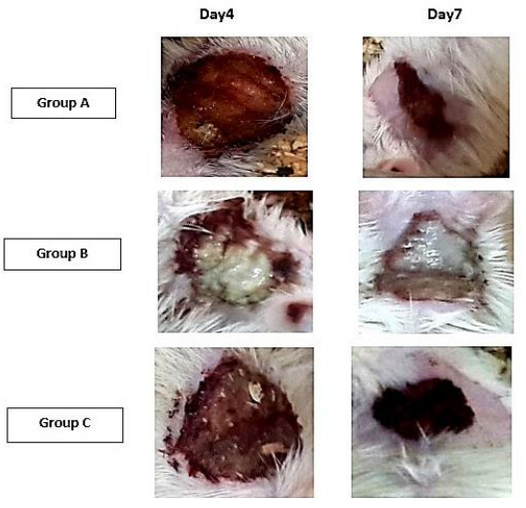

Fig. 6. *In vivo* wound healing abilities of recombinant and commercial lysostaphin.
Group A: Control group that were treated with recombinant lysostaphin. group B: Negative control group that were treated with just userine. Group C: Positive control group that were treated with commercial lysostaphin.

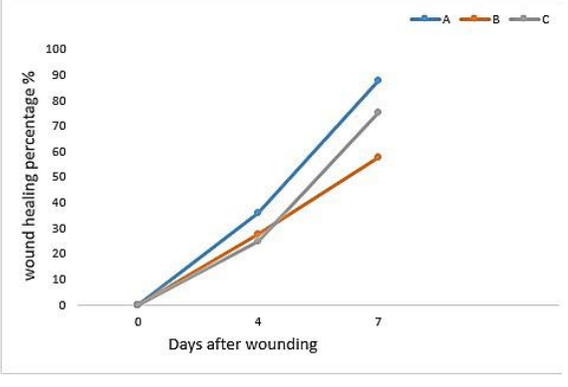

Fig. 7. Graphical representation of wound healing percentage during 7 days (A) Control group that were treated with recombinant lysostaphin (B) Negative control group that were treated with just userine. (C) Positive control group that were treated with commercial lysostaphin.

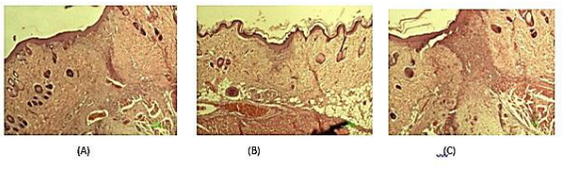

Fig. 8. Microscopic tissue area on the 7th day of healing course shows evidence of wound contraction with healing tissue (fibroblasts and blood vessel proliferation). Arrow notes the wound's surface and neovascular. (A): A group, (B): C group, (C): B group

DISCUSSION

Since lysostaphin has the ability to treat staphylococcal infections, this study intended to produce a mature form of the lysostaphin recombinant protein to be applied on the skin as cream. The produced lysostaphin recombinant ointment significantly improved the skin infection within a week.

Lysostaphin, as a bacteriocin, is an anti-microbial peptidoglycan hydrolase produced by *S. simulans* (19). Mature lysostaphin is a 246-amino acid protein with a molecular weight of 27 KD containing two separate domains:

1) N-terminal peptidase domain (PD) responsible for the catalytic activity of the protein (20).

2) C-terminal cell wall-targeting domain (CWT) (SH3b), which binds specifically to the *S. aureus* cell wall, breaks the peptide bond between two adjacent glycines in pentaglycine peptidoglycan peptide bridges in the cell wall of the *S. aureus*, and disturbs the osmotic rupture of *S. aureus.* Therefore, lysostaphin kills sensitive cells by hydrolyzing the cell wall (21).

Lysostaphin has also exhibited highly powerful and specific bacteriolytic activity against *Staphylococcus*, including Methicillin-resistant *S. aureus.* Thus, it could be a therapeutic agent to eradicate staphylococcal infections.

Farhang et al. found that, lysostaphin, owing to its anti-staphylococcal activity, is capable of cell lysis and killing the *S. aureus* cells in the nose of a laboratory animal (11). Kokai-Kun et al. also examined the therapeutic effect of lysostaphin on the systemic infection of the vancomycin-resistant clinical strains of *S. aureus* in a rat model and showed good efficacy of lysostaphin (22).

However, few studies have investigated the therapeutic and anti-staphylococcal effects of lysostaphin on the treatment of skin infections in animal models. In the present study, we also employed *in vitro* and *in vivo* methods to examine the therapeutic and anti-staphylococcal effects of lysostaphin on rat skin infections. Our results revealed that lysostaphin could heal wounds.

S. aureus causes various infections from skin infections to its spread and as a result systemic infections lead to organ failure and death (23). Owing to the emergence of antibiotic resistance, controlling this bacterium is highly difficult. The drug resistance in this group of pathogens requires research on new antibacterial compounds. Lysostaphin is one of these

anti-bacterial compounds. Lysostaphin is an efficient treatment to eliminate *S. aureus* from both blood and infectious organs. The structures of SH3bs from lysostaphin identify a groove between β1 and β2 strands as the pentaglycine binding site. In addition, the pentaglycine specificity of SH3b arises directly by steric deletion of Cβ atoms in the ligand and indirectly by selection of the main chain conformations that are easily accessible for glycine, not for other amino acid residues (24).

Dixon et al. tested the effectiveness of lysostaphin against stable renal abscesses in a rat model. They found that the compound significantly reduced the population of staphylococci in all rats (25). Patron and Climo also examined the effect of lysostaphin on the endocarditis caused by *S. aureus.* In their study, they utilized a rabbit with aortic valve endocarditis created by two clinical isolates of *S. aureus*, MRSA, and VISA. In the endocarditis caused by MRSA species, the animals were treated with either different doses of recombinant lysostaphin and vancomycin or a combination of both for three days. The results revealed that compared to the control group, the reduction of bacterial density with a combination of lysostaphin and vancomycin was greater than the use of each factor alone. Furthermore, regarding the endocarditis caused by VISA species, it was found that vancomycin was ineffective, and the aortic valve clearance rate was 43% in animals treated with a single dose of lysostaphin and 83% with twice-daily injection for three days. Their results also indicated that lysostaphin could be an effective treatment to eliminate *S. aureus* from the blood and the infected organs in mice (26).

In the present study, we explored the effectiveness of recombinant lysostaphin, as an anti-bacterial compound, in the improvement of skin infections caused by MRSA in laboratory animals.

The lysostaphin dosing used in our experiment was consistent with the one utilized in the MIC test, the lowest dose that could kill MRSA. Hence, 100 µg/mL dose of lysostaphin can significantly reduce the MRSA infection.

Although the serum half-life of lysostaphin is short (1 hour), the tissue half-life of lysostaphin is longer, and lysostaphin can maintain its lethal effect in tissues for a longer time. Accordingly, we applied lysostaphin cream to a bacterial wound every 12 hours. Our recombinant lysostaphin significantly killed the bacterial infection four days after infection, and the infection completely vanished, so that the wound healed fully after seven days.

Moreover, results of histological and microscopic studies on our animal model in three groups of samples (positive control, a case, and a sham) indicated that recombinant lysostaphin decreased necrotic tissue, collagen production, and infection and improved wound like commercial lysostaphin. Additionally, the infection caused by MRSA in the rat skin of the sham sample did not improve over time, but the positive control and case samples improved significantly seven days after using lysostaphin, demonstrating that recombinant lysostaphin, like commercial lysostaphin, had biological activity, decreased, and eventually eliminated bacteria through anti-bacterial activity. Owing to the significant effect of lysostafin on wound healing, this product can be used to create wound dressings to heal staphylococcal wounds.
Finally, it should be noted that various microorganisms are effective in wound infections, and the antimicrobial effect of lysostaphin on these microorganisms should be assessed in future studies.

Since lysostaphin has the ability to treat staphylococcal infections, this study aimed to produce the lysostaphin recombinant protein to be applied on the skin as cream. The produced lysostaphin recombinant cream significantly improved the skin infection within a week.

In conclusion, Our present data confirm that topically recombinant lysostaphin was effective in wound healing caused by the *S. aureus* infection.

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