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Evaluation of bacteriophage products against burn wound Methicillin-resistant *Staphylococcus aureus* (MRSA) infections

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ABSTRACT

Background: The major problem in the management of burn wounds are infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the major cause of infection in burn wounds. Antibiotic resistant bacteria around the world has become a major therapeutic challenge. Bacteriophages and their lysine are suggested as an antimicrobial alternative agent. Phage display technique is suggested for production of recombinant lysine by Nano carrier technology. The approach of this study was to evaluate the potential of recombinant Nano phage efficacy in MRSA burn wound infection in vivo.

Materials and methods: The 3rd degree burn wounds were induced in 54 rats and infected with MRSA ATCC 33591 via the topical route in four groups. Burn wound size was measured in 0, 14, 21, 28 days. The efficacy of Nano phage gel was assessed on the basis of percentage collagen deposition, granulation tissue, neovascularization, fibroblastic maturity, re-epithelization, and scar formation in rats following treatment in 14, 21, 28 days.

Results: The results showed that the percentage of wound size were 3 cm on base line day and the average macroscopic wound healing rates were increased in the prevention groups receiving the recombinant Nano phage gel and natural phage gel, in the treatment groups with secondary infection receiving the recombinant Nano phage gel and the natural phage gel, and in the two control groups respectively. The average microscopic wound healing rates were increased in the prevention groups receiving the recombinant Nano phage gel and natural phage gel, in the treatment groups with secondary infection receiving the recombinant Nano phage gel and the natural phage gel, and in the two control groups respectively.

Conclusions: In conclusion the recombinant Nano phage gel is efficacy to treat and prevent MRSA burn wound infection.

1. Introduction

The major problem in the management of burn wounds is their infection. Methicillin-resistant *Staphylococcus aureus* (MRSA) burn wound infection increase burn-related morbidity and mortality and is one of the major cause of sepsis in these patients. The prevalence of infections caused by MRSA has increased from 12 % in 1992 to 80.03 % in recent years. The mortality rate of MRSA infection in case of burn wound patients is much higher than Methicillin-sensitive *Staphylococcus aureus* (MSSA) (42 % and 18 % respectively) [1]. The widespread crisis and spread of antibiotic-resistant bacteria around the world has become a major therapeutic challenge [2]. The emergence of multi-drug resistant *S. aureus* worldwide has led to an increase interest in phage medicine as a possible alternative or at least supplementary approach to antimicrobial agents for management of some bacterial infections [3]. Bacteriophage is the natural viruses of bacteria that able to destroy

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bacterial cells. The purification from endotoxins of bacteriophages has been much improved their safety. There are some positive results for phage therapy which recommends the use of this option over antibiotics for treatment of bacterial diseases [4]. Unlike antimicrobial agents which may produce contraindications, phage therapy produces no or few unwanted effects when used for prevention and treatment. After phage therapy, the bacteriological analysis of wound secretions showed that *staphylococcus* strains decreased significantly [5]. Several case series have been reported that phage therapy could be used to improve burn wound treatment and prevent sepsis in these patients [3]. The efficacy of phage therapy depends on sensitivity and accessibility of bacterial host to the phage, phage titer, routes of phage administration, duration of course, and so on [3]. Potential beneficial effects of phage therapy are activity against MDR-pathogens, improved efficacy as compared with antibiotics, low side effects, preservation of the existing microbiome, possible effect on the inflammatory response and cost effectiveness. Phages encoded protein such as endolysin has proved their ability as a new alternative to antibacterial products [2]. The endolysin of the bacteriophages are highly evolved molecules that efficiently and quickly allow their progeny to be released from the host bacterium. These enzymes destroy peptidoglycan component of the bacterial cell walls [6,7]. The phage endolysin designated LysK isolated from the staphylococcal bacteriophage K has been shown to have potent antimicrobial activity against a range of *staphylococci* including MRSA. LysK contains two catalytic domains: a cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) domain, and an N-acetylmuramoyl-L-alanine amidase domain. Soluble CHAPk displayed at least a two-fold increase in lytic activity against *staphylococcal* cells *In vitro* [8]. It has been truncated to single catalytic domain, a cysteine, histidine dependent aminohydrolase/peptidase / (CHAPk). This domain, 18.6 kDa antibacterial enzyme has demonstrated retention of lytic activity and against *staphylococcal* biofilms [9]. The prospects for Nano biotechnology are facilitated by the enzymes' structures. Interestingly, by phage display technique, the peptide or protein gene are expressed so peptide or protein are displayed on the surface of Nano-carrier [10,11]. Passive and active targeting are caused by Nanocarrier technology. In passive targeting the recombinant endolysin penetrate to the depth of tissue, the effective dose is reduced, and there is no toxic effect on eukaryotic cells. Active targeting specifically lysis MDR strains and have no effect on microflora bacteria. The smart recombinant Nano phages are appropriate candidates as they specifically lysis MRSA with the least effective dose, have high penetrating power, and no side effects. They are also produced on a large scale at a moderate cost [12]. It is essential to conspire recombinant Nano phages to eliminate MRSA from colonized burn wounds. Hence, the present study was aimed to evaluate the potential of recombinant Nano bacteriophage efficacy in MRSA burn wound infection as a low-cost and effective treatment.

2. Materials and methods

2.1. Preparation of recombinant Nano phage

Previously in our study the CHAPK gene with 501bp was selected from reference sequence database ID, KY974323.1, in the NCBI. The CHAPK gene was ligated in T7Select vector arms in T7Select10-3b Cloning Kit (Novagen, U.S.A) with ligated reaction. The ligated reaction directly was added to packaging extract in T7Select10-3b Cloning Kit (Novagen, U.S.A). Finally, recombinant Nano phage were manufactured with titer 2×10^9 PFU/ml [13,14].

2.2. Antimicrobial determination of recombinant Nano phage in vitro

To investigate lytic activity recombinant Nano phage against MRAS (ATCC No.33591) turbidity Concentration assay and spot test were performed. Serial dilution of MRSA ATCC No. 33591 in LB medium (1.5×10^8 CFU/ml) were prepared in 7 tubes. The ratio of 1:1 diluted MRSA (ATCC No.33591) with recombinant Nano phages (2×10^9 PFU/ml) were incubated at 37 °C. The concentration of MRSA ATCC No.33591 was checked at interval 5 min in presence of recombinant Nano phage at OD_{600} nm. Similar to the aforementioned method natural bacteriophage (2×10^9 PFU/ml) was used as control group. Natural bacteriophage was previously isolated from sewage in Sari Bouali Sina Hospital, Mazandaran province in north of Iran [15–17].

3. Preparation of recombinant Nano phage gel

To prepare the plain gel, HPMC (5%) was dispersed in water and kept overnight. Then mixed under propeller homogenizer at 400 rpm to prepare a homogenous gel. To prepare recombinant Nano phage gel, 100 g of recombinant Nano phage (equal to 10^9 PFU/ml) was mixed with 900 g plain gel under propeller homogenizer at 400 rpm. To prepare natural phage-solution gel, lytic phage solution (equal to 10^9 CFU/ml) was mixed with 900 g plain gel under propeller homogenizer at 400 rpm [18].

3.1. Experimental protocol

3.1.1. Animals

Fifty-four female wistar rats, aged six months old, weighting 200 ± 10 g were prepared from institute of animal experimental studies at Mazandaran University of Medical Sciences in Sari, Iran. The rats were caged under controlled conditions of light, room temperature and humidity for a week prior to study. This study was approved by the ethical committee of Mazandaran University of Medical Sciences [19].

3.1.2. Burn wounds induction

The 3rd degree burn wounds were induced on shaved area of dorsal skin of the rats under anesthesia (intraperitoneal injection of 100/5 mg/kg ketamine/xaylazin) using hot plate sized 3 cm × 1 cm at temperature of 156° F or 69° C for 3 s. After 30 min, four groups were inoculated 0.1 ml of 0.5 McF, MRSA (ATCC 33591) solution (1.5×10^8 CFU/ml) [20,21]. The 54 rats individually were caged and divided in to 6 groups (N = 9) rats as follows:

Group A: Natural bacteriophage gel was prepared with 10^9 PFU/ml bacteriophage and was applied on burn wounds, twice a day.

Group B: Recombinant Nano phage gel was prepared with 10^9 PFU/ml and was applied on burn wounds, twice a day.

Group C: Natural bacteriophage gel was prepared with 10^9 PFU/ml bacteriophage and was applied on infected burn wounds, twice a day.

Group D: Recombinant Nano phage gel was prepared with 10^9 PFU/ml and was applied on infected burn wounds, twice a day.

Group E: base gel was prepared with HPMC (5%) and was applied on infected burn wounds, twice a day.

Group F: The infected rats that received no medication.

3.1.3. Burn wounds gross morphology assessment

Burn wound area in different groups on day 14, 21, 28 were measured with millimeter scale and percentage wounds contraction was calculated in terms of percentage change in initial wound size [36–37]. The percentage of wound size and recovery was calculated according to Eqs. (1) and (2) [21].

$$WC(\%) = \frac{\text{Initial wound size-specific day wound size} \times 100}{\text{Initial wound size } (300\ mm^2)} \quad (1)$$

$$\text{Percent of wound recovery} = 100 \_ \text{percent of wound area} \quad (2)$$

3.1.4. Burn wounds histological assessment

The three samples with dimensions of 3.5 cm × 2.5 cm wound tissues were taken of each group and were fixed in 10 % formalin that were sacrificed by spinal cord on day 14, 21, 28. Paraffinembedded

sections (5-μm thick) were prepared and were stained with hematoxylin & eosin. Light microscopy was used to evaluate collagen deposition, granulation tissue, neovascularization, fibroblastic maturity, re-epithelization, and scar formation [19,22].

3.1.5. Bacterial load in blood and burn wounds

To detect of the presence of MRSA bacteria in blood, on days 14, 21, 28 blood samples of rats in each group under anesthesia were removed and were cultured in 25 ml biphasic culture medium, then were incubated at 37° C overnight. To check the presence of MRSA bacteria on the surface of burn wounds with sterile swab were sampled and inoculated into Nutrient Broth, then were incubated at 37° C overnight. After 24 h the presence of MRSA was checked by using common laboratory tests [20].

3.2. Statistical analysis

The data were analyzed by SPSS 24 software using Two-way analysis of variance and Bonferroni post-hoc test.

4. Results

4.1. Determination the lytic activity of recombinant Nano phage in vitro

To investigate lytic activity of 2 × 10^9 PFU/ml recombinant Nano phage against MRSA ATCC No. 33591 turbidity concentration assay was used. The results shown after 5 min recombinant Nano phage 2 × 10^9 PFU/ml have lytic activity bactericidal effected against MRSA ATCC No. 33591 Fig. 1. The result of spot test showed lytic activity of recombinant Nano phage against MRSA ATCC No. 33591 with formation the zone of inhibition Fig. 2).

4.2. Gross morphology examination

The percentage of wound size were 3 cm on base line day for each rat in all the groups. On 28th day, the percentage of wound size were significantly decreased in (B) recombinant Nano phage gel (P < 0/01), (A) natural bacteriophage gel (P < 0/01 (D) recombinant Nano phage gel and infected burn wounds (P < 0/01), (C) natural bacteriophage gel and infected burn wounds (P < 0/01), (E) base gel and infected burn wounds (P < 0/01), (F) untreated group and infected burn wounds (P < 0/01) respectively (Fig. 3). The wound size was significantly lower in recombinant Nano phage gel (p < 0.01) and natural bacteriophage gel (P < 0/01) as compared to base gel and untreated group

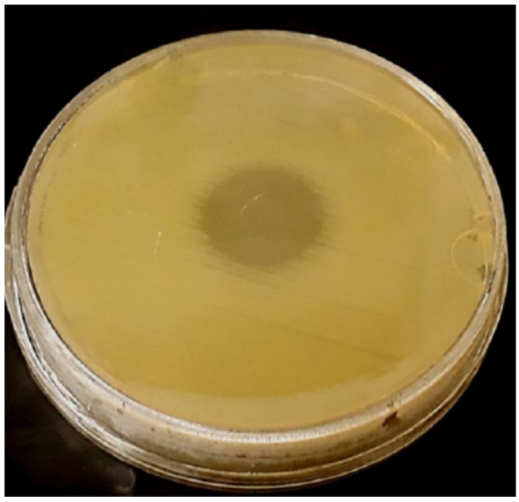

Fig. 2. Lytic activity of recombinant Nano phage against MRSA ATCC No.33591 by spot test.

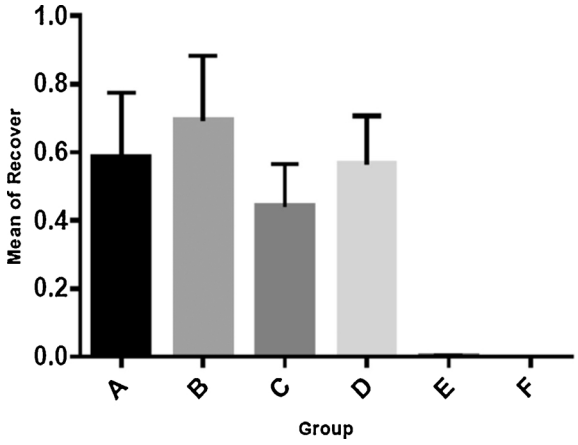

Fig. 3. The average macroscopic wound healing rates in the treatment groups On 28th day, were statistically significant P value < 0.01. A, Natural phage gel group; B, recombinant Nano phage gel group; C, Secondary infection & Natural phage gel group; D, Secondary infection & Recombinant Nano phage gel group; E, Secondary infection & base gel group; F, Secondary infection & untreated group.

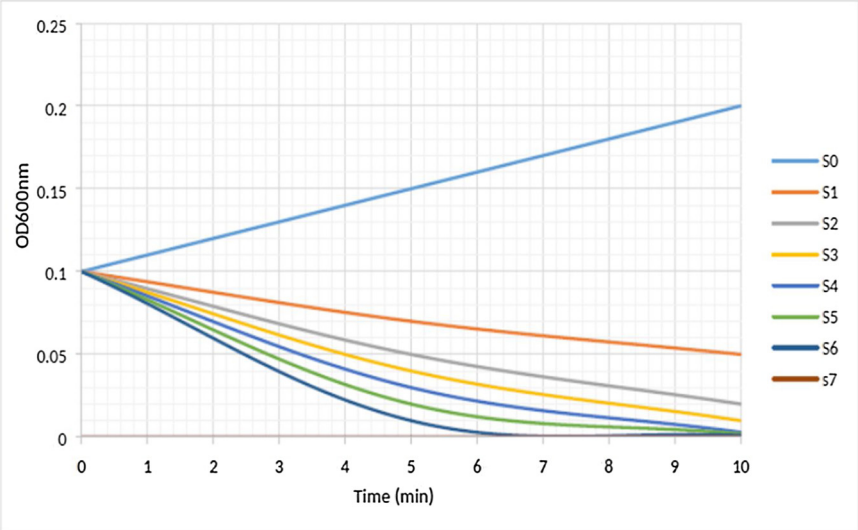

Fig. 1. Lytic activity of recombinant Nano phage against MRSA ATCC No. 33591 after 5 min.

Table 1
Percentage of wound healing after burn wound induction on 14, 21, 28 days of treatment in 6 groups (N = 9).

Groups	14 days	21 days	28 days
A	0.409 ± 0.052	0.527 ± 0.034	0. 823 ± 0.025
B	0.493 ± 0.012	0.660 ± 0.036	0.927 ± 0.021
C	0. 307 ± 0.028	0.450 ± 0.050	0.573 ± 0.064
D	0.409 ± 0.009	0.552 ± 0.020	0.733 ± 0.015
E	0.000 ± 0.000	0.000 ± 0.001	0.005 ± 0.005
F	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

A, Natural phage gel group; B, Recombinant Nano phage gel group; C, Secondary infection & Natural phage gel group; D, Secondary infection & Recombinant Nano phage gel group; E, Secondary infection & base gel group; F, Secondary infection & untreated group.

(Table 1).

4.3. Histopathological studies

The percentage of collagen deposition, granulation tissue, neovascularization, fibroblastic maturity, re-epithelization and scar formation on 28th day was significantly increased in (B) recombinant Nano phage gel, (A) natural bacteriophage gel, (D) recombinant Nano phage gel and infected burn wounds, (C) natural bacteriophage gel and infected burn wounds, (E) base gel and infected burn wounds, (F) untreated group and infected burn wounds respectively. Whereas the percentage of inflammation, angiogenesis was significantly lower in (B) recombinant Nano phage gel (P < 0/01), (A) natural bacteriophage gel (P < 0/01) as compared to (D) recombinant Nano phage gel and infected burn wounds (C) natural bacteriophage gel and infected burn wounds, (E) base gel and infected burn wounds, (F) untreated group and infected burn wounds. The data are summarized in (Figs. 4, 5).

4.4. Bacterial load in blood and burn wounds

The MRSA contamination was observed after 2, 14, 21, 28 days on wound surface and blood culture test in the untreated and gel base groups. MRSA contamination was not detected on days 14, 21, and 28 in the groups receiving the recombinant Nano phage gel (Table 2).

5. Discussions

This study used recombinant Nano phage to treat and prevent MRSA burn wound infection. The Nano phages were manufactured by phage display technique [10,12]. The hyperlytic part of endolysine CHAPK was fused to the carboxyl part of gp10B capsomere on the surface of T7 phages. The recombinant Nano phages exhibited bactericidal activity against the standard strain of MRSA ATCC 33591 after five minutes *In vitro*. Following that, lytic activity of the recombinant Nano phage gel against Methicillin-resistant *S. aureus* was studied under *In vivo* by inflicting third-degree burns in Wistar rats and infecting the burn wounds with the Methicillinresistant standard MRSA ATCC 33591.

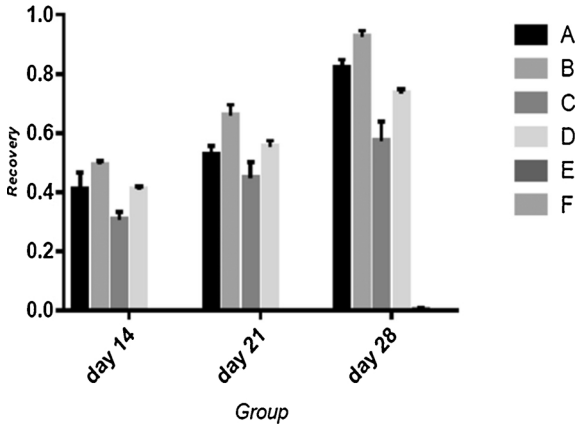

Fig. 4. The average microscopic wound healing rates in the treatment groups on 14, 21, 28 days, were statistically significant P value < 0.01. A, Natural phage gel group; B, recombinant Nano phage gel group; C, Secondary infection & Natural phage gel group; D, Secondary infection & Recombinant Nano phage gel group; E, Secondary infection & base gel group; F, Secondary infection & untreated group.

Bacteriophages have been used for a long time to treat infectious diseases [4,5]. For example, PhageBioDerm has been used in Georgia for treating wound infections caused by *S. aureus, Pseudomonas aeruginosa, Escherichia coli, Streptococcus,* and *Proteus*. Endolysine has been recently suggested as a new antimicrobial class for treating drug-resistant infections [23,24]. In this study, we used the Nano carrier technology by phage display technique to construct the recombinant lysine. In the phage display technique, the endolysine is displayed on Nano phage surface. Two features are defined for these Nano phages: active targeting and passive targeting. In active targeting, the smart recombinant Nano phages have specific anti-MRSA lytic activity, whereas in passive targeting no immune response is exhibited against lysine, having the lowest number of amino acids and low molecular weights. Moreover, since these recombinant phages are at nanoscale, their depth of penetration in tissue increases [12]. Survival percentage in the prevention groups receiving the recombinant Nano phage gel or the natural phage gel, and in the treatment groups including the groups with secondary infection and treated with the recombinant Nano phage gel or with the natural phage was 100 percent compared to the two control groups (the group with secondary infection treated with the gel base and the group with secondary infection that did not receive any treatment). However, in study Chopra et al. in 2016, survival rate in the group treated with 50 μg of the recombinant 10MR lysine was 35 % [1]. In the present study, MRSA were not detected on days 2, 14, 21, and 28 of the treatment on wound surface and on blood culture test in the prevention groups receiving the recombinant Nano phage gel and the natural phage. These bacteria were not present on wound surface and on blood culture test in the treatment groups including the group with secondary infection treated with the recombinant Nano phase gel. However, in the group with secondary infection treated with the natural phage these bacteria were detected on treatment days 2 and 14 but not on treatment days 21 and 28. The bacteria were present on wound surface and on blood culture test in the two control groups on days 2, 14, 21, and 28. However, it was reported in the study conducted by Chopra that *S. aureus* bacteria were present in skin, blood, liver, and spleen samples taken in the group treated with 50 μg endolysine MR-10 on treatment days 1, 3, and 5 but not on treatment day 7 [1]. In the present study, the average macroscopic wound healing rate in the prevention groups receiving the recombinant Nano phage gel or the natural phage gel increased on days 14, 21, and 28 by 49, 66, and 92 % and by 40, 52, and 82 %, respectively. The difference between two groups was statistically significant (P value < 0.01). The average macroscopic wound healing rate in the treatment groups with secondary infection receiving the recombinant Nano phage gel or the natural phage gel increased on days 14, 21, and 28 by 40, 55, and 73 % and by 30, 45, and 57 %, respectively. The difference between this two groups were statistically significant (P value < 0.01). The average macroscopic wound healing rates in the two control groups with secondary infection receiving the gel base or not receiving any treatment were 0.5 and 0 %, respectively. This indicated the effect of the recombinant Nano phage gel and the natural lytic gel on burn wound healing. However, Chopra reported that the macroscopic burn wound healing rate in the treatment group receiving 50 μg of endolysine MR-10 was 49 % on treatment day 12. In the present study, the average pathological burn wound healing rates based on clinical indices including acute inflammation, chronic inflammation, angiogenesis, fibroblast maturation, collagen deposition, granulation, and re-

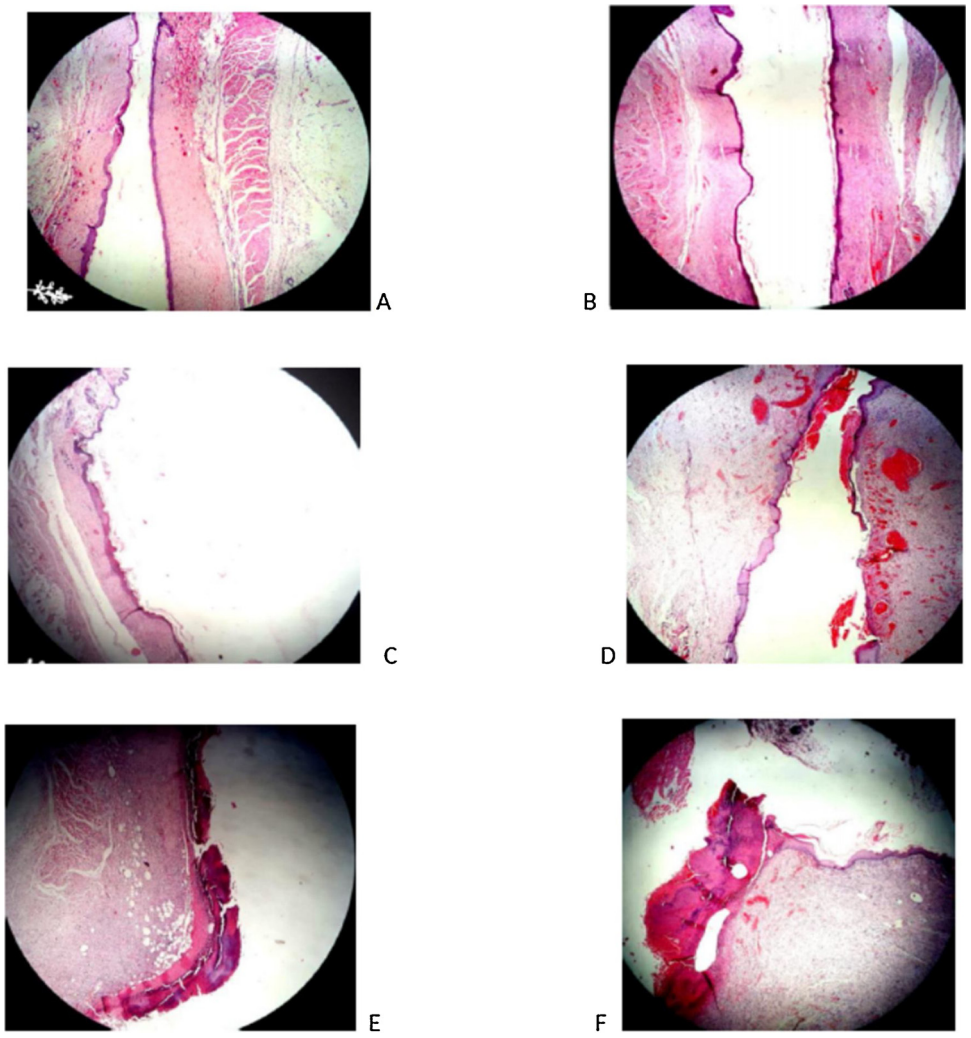

Fig. 5. Morphological changes of the rat's skin lesion 28th days after burn wounds induction. Hematoxylin-eosin, 40 × . (A) Rats treated with natural bacteriophage gel;(B) Rats treated with recombinant Nano phage gel;(C) Infected rats and treated with natural bacteriophage gel;(D) Infected rats and treated with recombinant Nano phage gel; (E) Infected rats and treated with base gel; (F) Untreated infected rats.

Table 2
The assessment of colonization MRSA ATCC No.33591 on surface of burn wounds.

Time 28 days	Time 21 days	Time 14 days	Groups
Negative	Negative	Negative	**A**
Negative	Negative	Negative	**B**
Negative	Negative	Negative	**C**
Negative	Negative	Negative	**D**
Positive	**Positive**	**Positive**	**E**
Positive	**Positive**	**Positive**	**F**

A, Natural phage gel group; B, Recombinant Nano phage gel group; C, Secondary infection & Natural phage gel group; D, Secondary infection & Recombinant Nano phage gel group; E, Secondary infection & base gel group; F, Secondary infection & untreated group.

epithelization were studied. The average pathological burn wound healing rates in the two prevention groups one of which received the recombinant Nano phage gel and the other the natural phage gel increased on days 14, 21, and 28 increased by 65, 73 and 85 %, and 56, 66, and 80 %, respectively. This difference between the two groups was not statistically significant. The average pathological wound healing rates in the two treatment groups with secondary infection one receiving the recombinant Nano phase gel and the other the natural lytic phage gel increased on day 14, 21, and 28 by 50, 56, and 70 % and by 46, 60, and 65 %, respectively. This difference between the two groups was not statistically significant. The average macroscopic wound healing rates in the two control groups with secondary infection one receiving the base gel and the other receiving no treatment were 16 % and 0 %, respectively. This difference between the two control groups was not statistically significant. However, the pathological symptoms of burn wound healing in the control group treated with 50 μg of the recombinant endolysine MR-10 was reported on treatment day 22 in the study conducted by Chopra.

In a case report, Totte et al. in 2017, used the recombinant lysine CHAPK (called Staphefekt SA. 100) topically to treat acne vulgaris, eczema, and folliculitis. They reported the lowest bacterial concentration and no bacterial resistance to the recombinant endolysine CHAPK in patients treated with Staphefekt SA. 100. Therefore, this recombinant Nano phase gel could be used against burn wound infection in clinical trials. In the present research, toxicity evaluation of the recombinant Nano phage gel using the MTT assay was not performed [25].

6. Conclusions

According to our latest review, this is the first time that use of a recombinant Nano phase gel is suggested for treating and preventing

burn wound infection. Recombinant Nano phages manipulated by using Nano carrier technology exhibit lytic activity against MRSA in a completely smart manner, and the recombinant lysine CHAPK has the lowest molecular weight and the highest permeability and distribution coefficients. According to the *In vivo* results, the highest average macroscopic and microscopic burn wound healing percentage in the treatment group receiving the recombinant Nano phage gel was observed on treatment day 28. Moreover, MRSA bacteria were not detected on burn wound surface or on blood culture test in this group.

Consent for publication

All authors Consent for publication.

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Declaration of Competing Interest

All authors declared no conflict of interest in present manuscript.

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