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Evaluation of *in-situ* gel-forming eye drop containing bacteriophage against *Pseudomonas aeruginosa* keratoconjunctivitis *in vivo*

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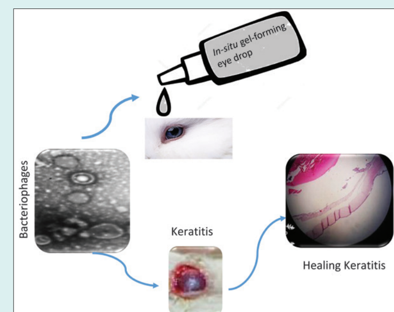
Abstract

Introduction: Eradication of *Pseudomonas aeruginosa* has become increasingly difficult due to its remarkable capacity to resist antibiotics. Bacteriophages have been suggested as an alternative treatment for bacterial infections.

Methods: *In-situ* gel-forming eye drop containing phage against *P. aeruginosa* keratoconjunctivitis was prepared. The *Cystoviridae* phage was formulated as *in-situ* gel-forming formulation which is a solution formulation but turns into gel when it contacts the eye. Therapeutic effectiveness of the *in-situ* gel forming formulation was evaluated by histological examination on day 12 post-infection.



Results: The viscosity of selected formulation increased when it was instilled into the eye. The histological results showed edema, abscesses, and destruction of the stromal structure of cornea in groups where no *in-situ* gel-forming formulation was used. In the group where *in-situ* gel forming formulation was used, *re-epithelialization* and normal corneal structure were observed.

Conclusion: *In-situ* gel-forming ophthalmic formulation containing phage can be effective in the treatment of *P. aeruginosa* keratoconjunctivitis.

Introduction

Pseudomonas aeruginosa is associated with many types of ocular infections such as conjunctivitis, keratitis, and eyelid disorders. Keratitis is the most serious eye infection and the main cause of corneal blindness. Keratitis can be caused by mechanical trauma, scratches, medical contact lenses, cosmetic contact lenses, and contact with contaminated water. About 30 000 cases of microbial keratitis are reported in the United States annually.[1,2]

Pseudomonas aeruginosa possesses a high level of intrinsic resistance to most antibiotics due to its restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell, and production of antibiotic-inactivating enzymes such as β-lactamases.[3-8] Bacteriophages offer an alternative treatment for bacterial infections. Bacteriophages are a type of virus that can infect bacteria. They can specifically destroy the host bacteria without affecting the mammalian cells and the microbiota. Furthermore, bacteriophages are self-replicating and replicate at the site of infection, therefore, it is simple and inexpensive to produce.[9-13]

It has been shown that phage therapy can successfully

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be employed for the treatment of eye infections.[9-11] In a study, eye drops containing bacteriophage have been used three times a day on 28 conjunctivitis patients where the patients have shown signs of recovery. The US-based Phage Biotech Ltd has announced the production of topical phages as a treatment for keratitis caused by contact lenses.[10]

Although drug delivery to ocular mucosa to create the local effect is an excellent idea, it is also associated with many challenges and hurdles. Generally, conventional topical ophthalmic products are not suitable for treating eye infections. This is due to poor bioavailability of drugs[14] when it is used through the eye and also due to a shorter residence time of drop solution on the eyes. Typically, the bioavailability of eye drops is less than 5% but it depends on the physicochemical properties of the drug.[15] It seems increasing the viscosity of eye drop solutions may increase the residence time of the formulation and enhance the bioavailability which has been discussed in a review article.[16] In addition, the presence of barriers in the eye can limit drug absorption via these drug delivery systems.[17] In another study, the *in-situ* gel-forming estradiol eye drops have been used for the development of safe, sterile, and stable formulations for cataract prevention and helped elucidate the mechanisms by which estrogen protects lens transparency.[18]

In the current study, an *in-situ* gel-forming system was suggested for the effectiveness of phage therapy in the eye infection. In this novel drug delivery system, the drug was mixed with various types of polymers where under certain conditions such as pH, temperature, solvent, and ions the polymers form a gel. In the current research, pH was the only factor used to convert the solution to gel. *The in-situ* gel-forming system reduces the frequency of dosing, increases the contact time of the drug in the eyes, and increases patient compliance.[19]

Based on the above descriptions, the purpose of this study was to produce an *in-situ* gel-forming eye drop formulation containing phage with new features such as the long-term release of bacteriophage and increasing the durability of bacteriophage in the eye to provide an improved bioavailability for the eye product, and subsequently lower the dose of active phage ingredient. The *in-situ* gel-forming eye drop formulation containing phage can be used for the subsequent treatment of *P. aeruginosa* keratoconjunctivitis.

Materials and Methods

Materials

In the microbial tests, chocolate agar and blood agar were purchased from Merck, Germany. Kirby–Bauer technique was performed by antibiotic discs and all discs including meropenem (10 µg), cefotaxime (30 µg), gentamycin (10 µg), and ciprofloxacin discs (10 µg) were purchased from Padtan Teb, Iran. Luria-Bertani (Quelab, USA), agarose (ACROS, Belgium), ammonium acetate (ACROS, Belgium), uranyl acetate (ACROS, Belgium) were used for the isolation and characterization of bacteriophage. For the preparation of the *in-situ* gel-forming system, sodium phosphate, sodium chloride, methylparaben, propylparaben, sodium carboxymethyl cellulose (NaCMC), sodium bicarbonate (NaHCO3), calcium chloride-2 H2O, KCl, hematoxylin and Eosin were obtained from Merck, Germany.

Preparation of bacterial strains

Pseudomonas aeruginosa was provided by Pasteur Institute of Iran, Tehran. It was cultured on the chocolate agar and blood agar, followed by overnight incubation at 37°C. To determine antibiotic susceptibility test, Kirby–Bauer technique was performed by antibiotic discs namely meropenem (10 µg), cefotaxime (30 µg), gentamycin (10 µg), and ciprofloxacin (10 µg).[20,21]

Isolation and purification of bacteriophage

Sewage sample (100 mL) was incubated for 24 hours at 37°C in a shaker incubator. The sample was centrifuged at 11 000 × g for 15 minutes followed by filtration of the supernatant through a 0.22 µm syringe filter at the sterile conditions and room temperature.[22,23]

Spot test

The overnight cultured *P. aeruginosa* (100 µL) was inoculated in a top agar and was poured into the bottom agar. Then the supernatant of bacteriophage (10 µL) was poured over the solidified agar followed by incubation at 37°C overnight. The formation of the inhibition zone was checked.[22,23] After 24 hours, the formation of the inhibition zone showed the isolation of phage and its lytic activity against bacteria.

Double-layer plaque assay (DLA assay)

Luria-Bertani (Quelab, USA), 900 µL, was added to 8 sterile tubes. Then 100 µL of phage was added to tube no. 1 and was vortexed. After vortexing the tube, 100 µL from tube no. 1 was taken and added to the tube no. 2. This procedure was repeated until tube no. 8.

Tube no. 9 (900 µL of *P. aeruginosa*) and tube no. 10 (900 µL of Luria-Bertani) were selected as the positive and negative controls, respectively. Then, 200 µL from each of the diluted phage was added to 200 µL *P. aeruginosa* (1.5× 10^8 CFU/mL). The mixture was added to the top agar followed by adding the top agar to the bottom agar. The plates were incubated overnight at 37°C.[22,23]

To calculate the plaque-forming unit (PFU), the following equation was used:

Plaque forming units (PFU) = the number of plaques×10×the inverse of the dilution factor

Transmission electron microscopy (TEM) study

The phage was centrifuged at 25 000×g for 60 minutes. To concentrate the bacteriophage, the phage was washed

in 0.1 M neutral ammonium acetate. The phage was deposited on carbon-coated copper grids and was stained by 2% uranyl acetate (pH 4-4.5). The phage was observed on a Zeiss EM 900 TEM at 150 kV.

Preparation of in-situ gelling system

Sterile isotonic phosphate buffer (pH 7.4) was prepared using 0.160 g monobasic sodium phosphate solution, 0.758 g dibasic sodium phosphate solution, and 0.44 g sodium chloride in 100 mL distilled water. To prepare the *in-situ* gel-forming system of bacteriophage, 0.05263 g methylparaben and 0.01052 g propylparaben were dissolved in 95 mL of hot phosphate buffer followed by the addition of 5 mL of bacteriophage (10^{10} PFU/mL) at 25°C under stirring conditions using a magnetic stirrer. In the end, 3 g of pectin and 0.5 g of sodium carboxymethyl cellulose (NaCMC) were added to the solution and allowed the polymers to hydrate fully overnight. The solution was mixed with a mechanical homogenizer after 24 hours. The procedure was performed under a laminar flow hood. The placebo was made using the same procedure without bacteriophage.[24]

Viscosity measurement and rheological behavior study

The viscosity of the prepared formulation was measured by Brookfield viscometer (Brookfield, DV-II +, USA) using spindle S5 at pH 7.4 before and after adding simulated tear fluid (STF) to the solution (2:5 ratio). The viscosity of the formulation was determined at different speeds (5, 10, 20, 50, 100 rpm). The temperatures of STF and the solution were 37°C and 25°C respectively. The appearance and clarity of the formulation before and after gelation was also checked visually under the light.

Gelling capacity studies

The gelling capacity was determined by adding 100 µL of *in-situ* gel-forming system in a test tube containing 2 mL of STF. The test tube was equilibrated at 37 ± 1°C and the formation of the gel was visually assessed. The time needed for the formation of gel and also dissolving the gel was recorded.

In vitro gelation study

The strength of the gels was evaluated by placing a drop of polymeric solution in a beaker containing 50 mL of freshly prepared STF equilibrated at 37°C. The composition of STF contained NaCl 0.680 g, sodium bicarbonate (NaHCO3) 0.220 g, calcium chloride dihydrate 0.008 g, KCl 0.14 g, and purified water q.s. 100.0 mL.

Sterilization and microbial test

The finished formulations were sterilized by autoclaving at 121°C and 15 Pa for 20 minutes. The sterilized formulations were stored in a refrigerator until use. In addition, 2 µL of the sterilized formulation was aseptically transferred to the fluid thioglycolate medium (20 mL) and soya bean-casein digest medium (20 mL), separately. The inoculated media were incubated for not less than 14 days at 30–35°C in the case of fluid thioglycolate medium and 20– 25°C in the case of soya bean-casein digest medium.

Experimental model of P. aeruginosa keratitis

Thirty-five New Zealand male rabbits each weighing around 2 kg were purchased from Pasteur Institute, Karaj, Iran. The rabbits were caged under controlled conditions of light, room temperature, and humidity for a week before the investigation.

The rabbits were anaesthetized with an intramuscular injection of ketamine: xylazine (30 mg/kg). The left eye of rabbits was scratched by a 25-gauge sterile needle. Bacterial corneal infection was induced by *P. aeruginosa* suspension (1.5 ×10^8 CFU/mL) 30 minutes after scratching the eye. The Rabbits were divided into five groups with 7 rabbits in each group (the total number of rabbits was 35). These groups were designated as group A (treatment; an *in-situ* gel-forming eye drops containing 10^{10} PFU/mL phage), group B (placebo), group C (positive control; povidone-iodine 5%), group D (negative control; the left eye was scratched and infected), and group E (non-treatment; the left eye was scratched and non-infected).

Histological examination

To evaluate the effect of the formulations on the treatment of the infected eye, histological examination was performed after 12 days. The rabbits were sacrificed by chloroform. The eyes were harvested and fixed in 4% formalin. The eyes were embedded in paraffin and the sections were stained with hematoxylin and eosin. The samples were investigated in terms of inflammation, angiogenesis, corneal destruction, infection, re-epithelialization, and conjunctivitis.[20, 21]

Statistical analysis

Fisher exact test was used to show the significance of the data obtained. To this end, SPSS 23 software was used.

Results and Discussion

Characterization of bacteriophage

In our study, *P. aeruginosa* was confirmed as a host cell by conventional microbial methods shown in Table 1. The Kirby-Bauer technique showed that *P. aeruginosa* is resistant to meropenem (10 µg), cefotaxime (30 µg), gentamycin (10 µg), but sensitive to ciprofloxacin (10 µg).

To determine the phage host and the lytic activity of phage, the spot test was performed. The formation of the inhibition zone was observed after 24 hours (Fig. 1). The phage titer was calculated by DLA assay (10^{10} PFU/mL).

The morphology of phage was investigated by TEM and is shown in Fig. 2. As the phage was spherical (80-85 nm) in shape with a lipid membrane around the capsomere, thus the phage can be classified into the *Cystoviridae* family (Fig. 2). The *Cystoviridae* phage (10^{10} PFU/mL)

Table 1. Specifications of *Pseudomonas aeruginosa*

Test	Results
Gram- staining	Gram-negative
Simmons' citrate agar	Positive
Catalase test	Positive
Oxidase test	Positive
TSI agar test	k/k
MR-VP tests	Negative

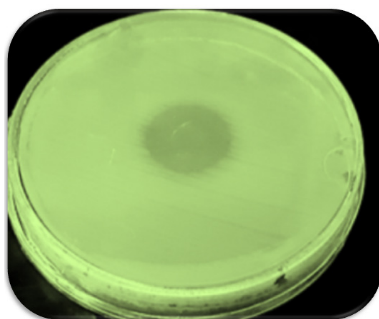

Fig. 1. Formation inhibition zone in spot test.

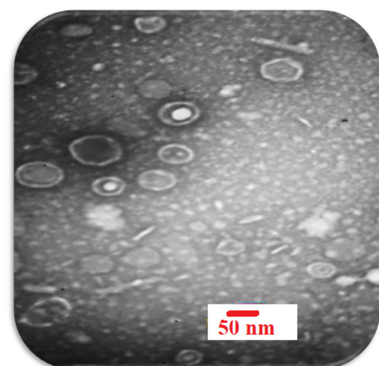

Fig. 2. Electron micrographs of *P. aeruginosa* phage belonging to the Cystoviredae family. With 2% uranyl acetate (pH = 4-4.5) was stained. (voltage 150 kV, the scale bar 50 nm).

was incorporated to *in-situ* gel-forming eye formulation. In 2016, Furusawa et al isolated *ϕR12, ϕR 12-1, ϕR 12-3, ϕR 26, ϕR 50,* and *ϕR 18* phages from sewage treatment plants at Sapporo and Ebetsu, Japan. The *Myoviridae* and *Podoviridae* phages (10^9 PFU/mL) were formulated as an eye drop.[25]

Characteristics of in-situ gel-forming system

In this study, an *in-situ* gel system was used. The selected formulation was a light yellow and clear solution with a pH of 7.4. The rheological study showed that the eye solution followed pseudoplastic behavior that is the reduction in viscosity with an increase in shear rate (Table 2). For example, when the solution was stirred at 5 rpm the viscosity was 80 cP, whereas this viscosity reduced to 24 cP when the stirring rate increased to 100 rpm (Table 2). The same pattern was obtained for the final formulation when the eye solution was mixed with the phage formulation. It was interesting to note that when the eye solution was added to the phage formulation, the viscosity of the final formulation increased (Table 2). For instance, at 5 rpm the viscosity of eye solution was 80 cP and this viscosity increased to 160 cP when the eye solution was added to the phage formulation. The same pattern was obtained for all other stirring rates (Table 2).

Because of the *in-situ* gel-forming system over the administration of the eye drop, it is expected that the effectiveness of bacteriophage and its contact time in the eyes should increase. When the eye drop is instilled into the eye, it becomes a gel within a couple of seconds and remains in gel state for around 10 minutes. The main factor for the formation of the gel is the presence of pectin in the formulation and calcium ions in the eye secretion. Pectines are from a polysaccharide family, where the polymer backbone mostly consists of α-(1,4)-D-galacturonic acid residues. Low methoxy pectines which are linked to the degree of esterification <50% can form a gel in aqueous solution in the presence of free calcium ions, that crosslink with galacturonic acid chains and enhances the viscosity.

The water solubility of pectin is one of the important advantages of the *in-situ* gel-forming formulation as there is no need to use organic solvents in the formulations. The *in-situ* gel-forming of pectin induced by calcium ions existing in lacrimal fluid has been reported in a US patent.[24] In addition, pectin-based *in-situ* gel-forming formulation can prolong drug release from the formulations.

The microbial growth test ruled out any microbial growth in the sterilized ophthalmic *in-situ* gel-forming formulations.

Histopathological examination

The results of eye histopathology showed the highest

Table 2. Viscosity of *in-situ* gelling solution at different speeds

Spindle number	Speed (RPM)	In-situ gelling solution		In-situ gelling solution after adding STF	
		Torque (%)	Viscosity (cP)	Torque (%)	Viscosity (cP)
5	5	0.1	80	0.2	160
5	10	0.2	60	0.2	80
5	20	0.2	40	0.2	50
5	50	0.4	32	0.4	35
5	100	0.6	24	0.7	28

destruction of the stromal structure of the cornea, edema of the corneal stroma, and large neutrophilic abscesses in the groups B, C, D, E on the 12th day. However, when the *in-situ* gel-forming eye drop formulation containing bacteriophage was used, the corneal structure was observed to be normal (Fig. 3).

The inflammation, angiogenesis, and abscess were significantly at the highest levels in the control group and placebo group, whereas inflammation, angiogenesis, abscess, and conjunctivitis were significantly at the lowest levels in the *in-situ* gel-forming eye drop containing bacteriophage ($P<0.001$) (see Fig. 3 and Table 3). In contrast, the inflammation, angiogenesis, and abscess were observed in the placebo group which data indicated that the base gel (plain gel) was not effective in the treatment of keratitis.

The histopathological results showed the highest conjunctivitis in the groups B, C, D, E whereas this was not the case when the *in-situ* gel-forming eye drop containing bacteriophage was used (Table 3). In Table 4, the results showed the highest re-epithelialization when the *in-situ* gel-forming eye drop containing bacteriophage was employed ($P < 0.001$).

The antimicrobial properties of bacteriophages incorporated in *in-situ* gel-forming eye drop may also influence the process of scratch and infection healing.[20,25] These data indicated antimicrobial properties of bacteriophages against bacterial host. Bacteriophages have lytic activity by the production of endolysin, which are responsible for the degradation of peptidoglycan - the main structural component of the bacterial cell wall.[12,13,22,23,26]

It has been shown that the antibacterial activity of phages against eye infection was positive in mouse models.[26] In their study, the number of viable bacteria in the infected corneas which were treated with *Myoviridae* and *Podoviridae* phages (10^9 PFU/mL) was determined and the result has shown that the phage was effective up to 3 hours after infection.[25] In the study carried out by Furusawa et al, the eye of 8-week-old C57BL/6 male mice was infected by 5 µL of *P. aeruginosa* NE-126 (10^4 CFU). After initial infection, 10^9 PFU of phages in 5 µL was applied to the corneal surface at 30 minutes, 1, 3, 6 and 12 hours. The results showed the administration of phage mostly eradicated the pathogen within 3 hours after infection.[25] But the phages did not prevent bacterial growth at 6 and 12 hours post-infection since *P. aeruginosa* invaded the deep corneal stroma and phages could not

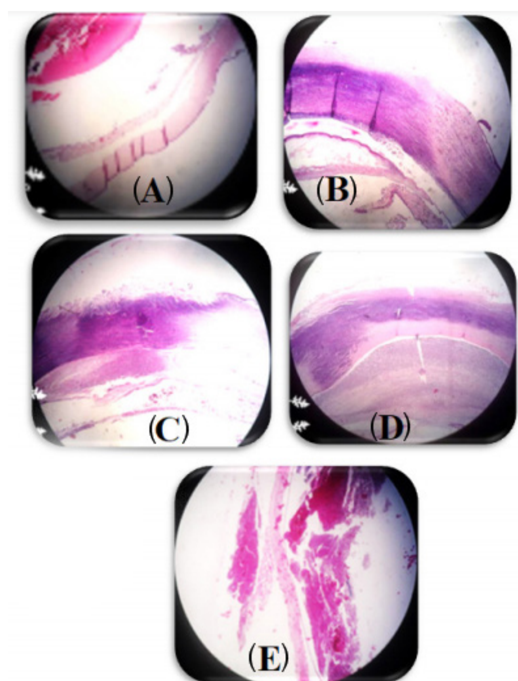

Fig. 3. Morphological changes of the rabbit's eye lesion 12th days after scratched induction. The hematoxylin & eosin, ×40. (A) rabbits treated with an *in-situ* gelling eye drops containing 1010 PFU/mL phage; (B)rabbits treated with an in-situ gelling eye drops without phage; (C) rabbits treated with Betadine 5%; (D) untreated infected rabbits ;(E) untreated and non-infected rabbits.

Table 3. Conjunctivitis of the cornea on 12 days of treatment in 5 groups (n = 7)

Conjunctivitis		Phage eye drop	Betadine	Placebo	Scratched and infected eye	Scratched and non-infected eye	*P* value
	Count	7	0	0	0	0	
No	% Within group	100.0%	0.0%	0.0%	0.0%	0.0%	<0.001
	% Of total	20.0%	0.0%	0.0%	0.0%	0.0%	
	Count	0	3	0	0	0	
+	% Within group	0.0%	42.9%	.0%	0.0%	0.0%	<0.001
	% Of total	0.0%	8.6%	.0%	0.0%	0.0%	
	Count	0	3	2	0	0	
++	% Within group	0.0%	42.9%	28.6%	0.0%	0.0%	<0.001
	% Of total	0.0%	8.6%	5.7%	0.0%	0.0%	
	Count	0	1	5	7	7	
+++	% Within group	0.0%	14.3%	71.4%	100.0%	100.0%	<0.001
	% Of total	0.0%	2.9%	14.3%	20.0%	20.0%	

Table 4. Re-epithelization of the cornea on the 12th day of treatment in 5 groups (n = 7)

Reepithelization		Phage eye drop	Betadine	Placebo	Scratched and infected eye	Scratched and non-infected eye	*P* value
No	Count	0	4	7	7	7	
	% Within group	0.0%	57.1%	100.0%	100.0%	100.0%	<0.001
	% Of total	0.0%	11.4%	20.0%	20.0%	20.0%	
+	Count	0	3	0	0	0	
	% Within group	0.0%	42.9%	0.0%	0.0%	0.0%	<0.001
	% Of total	0.0%	8.6%	0.0%	0.0%	0.0%	
+++	Count	7	0	0	0	0	<0.001
	% Within group	100.0%	0.0%	0.0%	0.0%	0.0%	
	% Of total	20.0%	0.0%	0.0%	0.0%	0.0%	

infiltrate there.

In another study, the eye of eight-week-old female C57BL/6 mice was infected by 5 μL of *P. aeruginosa* PA33 (5×10^6 CFU). After the initial infection, 5×10^8 PFU of phages in 5 μL was applied to the corneal surface on days 1, 3 and 5. The results showed only slight or focal corneal opacities and the lowest bacterial load on day 5 post-infection. The histopathological results showed that the corneas of non-treated mice exhibited denuded epithelium and central thinning. In addition, edema of the corneal stroma, large neutrophilic abscesses, and the presence of numerous inflammatory cells in the cornea were observed, but in phage-treated mice, a normal corneal structure on day 5 post-infection was observed.[20]

Compared to the published articles,[20,25] in our study, the *in-situ* gel-forming system is one of the promising approaches to improve the retention time of drugs on the ocular surface. The *in-situ* gel-forming system was developed to increase the ocular residence time, drug penetration across the ocular barriers, and ophthalmic bioavailability.

The potency of bacteriophages has been assessed for the treatment of keratitis in previous studies.[9-12,22,23] Proskurov reported a good clinical outcome in 17 patients with conjunctivitis and blepharitis who were treated with phage eye-drops in 1970.[26] Slopek reported the effect of phage therapy for recovery of patients with conjunctivitis between 1981 and 1986.[27] Kilasonia and Karanadze described the successful treatment of 32 children with acute bacterial conjunctivitis.[28]

In this study, the proposed *in-situ* gel-forming eye drop containing phage showed an excellent outcome for the treatment of *P. aeruginosa* keratoconjunctivitis. The *in-situ* gel-forming technique can be used to produce bacteriophage eye products with new attributes such as prolonging the release of bacteriophage, increasing the durability of bacteriophage in the eye, hence lower dose of active phage ingredient. Furthermore, this technique provides a better bioavailability for the products in the treatment of eye infections.

Research Highlights

What is the current knowledge?

√ The phage drop can be used for treatment of *P. aeruginosa* keratoconjunctivitis.

What is new here?

√ The *in-situ* gel-forming eye drop containing bacteriophage with new attributes such as the long-term release of bacteriophage and increased durability of bacteriophage in the eye. These features can lead to the use of a lower dose of active phage ingredient in the formulation. In addition, this technique provides a better bioavailability of the products for the treatment of eye infections.

Conclusion

In this study, *in-situ* gel-forming eye drop formulation containing phage for the treatment of *P. aeruginosa* keratoconjunctivitis was developed. The results proved that the *in-situ* gel-forming technique can be used to produce bacteriophage eye products with new features such as prolonging bacteriophage release and increasing the durability of bacteriophage in the eyes. These features can lead to the use of a low dose of active phage ingredient in the formulation. Moreover, this type of formulation can improve the performance of the products for the treatment of eye infections.

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Ethical statement

This study was approved by the Ethics Committee of Mazandaran

University of Medical Sciences, Sari, Iran (IR. MAZUMS.REC.1397.1333).

Competing interests
The authors declare that they have no conflict of interests.

Authors' contribution
GR, MSR: experiment design. MS, SSR, ME: formulation design. GR, AN, MSR, MS: supervision, writing and reviewing. MM: data analysis. M GH: histopathological analysis. GR, ME, PM, RP: experimental performance.

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