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Research Paper

Novel mucoadhesive polysaccharide isolated from *Bletilla striata* improves the intraocular penetration and efficacy of levofloxacin in the topical treatment of experimental bacterial keratitis

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Abstract

Objectives The objective of the present study was to evaluate a novel mucoadhesive polymer extracted from *Bletilla striata* for ocular delivery of 0.5% levofloxacin in rabbits, and to determine its improved efficacy against experimental keratitis.

Methods *B. striata* polysaccharide (BsP) was subjected to cell cytotoxicity and ferning tests. The pharmacokinetics and bioavailability of topically applied 0.5% levofloxacin-BsP eye drops was investigated and compared with 0.5% levofloxacin eye drops (Cravit). Experimental *Staphylococcus aureus* keratitis was induced and treated with levofloxacin or levofloxacin-BsP eye drops.

Key findings BsP markedly increased the proliferative capacity of a human corneal endothelial cell line. The ferning test showed that BsP exhibited optimal performance as a tear fluid. The polysaccharides significantly increased intra-aqueous penetration and corneal accumulation in rabbits. Treatment with levofloxacin-BsP reduced the number of organisms more significantly than eye drops containing levofloxacin alone.

Conclusions BsP appears to be a promising candidate as a vehicle for topical ophthalmic drug delivery, especially for antibiotics.

Keywords bacterial keratitis; *Bletilla striata*; levofloxacin; pharmacokinetics; polysaccharide

Introduction

Staphylococcus aureus is a major cause of bacterial keratitis, a sight-threatening condition. *S. aureus* ocular infections can cause severe inflammation, pain, corneal perforation, scarring, and loss of visual acuity.[1,2] Successful treatment of keratitis requires prompt diagnosis and the initiation of antibiotic therapy appropriate for the infectious organism, with antibiotics often administered via eye drops. However, with the increasing and widespread use of topical antibiotics, especially fluoroquinolone antibiotics, there is justifiable concern over the emergence of resistant organisms.[3] Improving the efficacy of antibiotics against common and virulent pathogens presents a therapeutic challenge, and failing to do so presents the risk for more serious infections. In the recent ophthalmic literature, several studies have reported increased in-vitro resistance among bacteria-causing keratitis to antibiotics commonly used in their geographic regions.[4,5] The severity of some strains of bacterial keratitis highlights the importance of rapidly attaining high drug concentrations at the site of infection. Drop regimens may fail to produce therapeutically active drug levels in ocular compartments because continuous tear flow reduces the bioavailability of topically applied antibiotics and the corneal epithelium acts as a barrier against drug penetration. Several drug delivery systems have been developed to prolong the contact time between antimicrobial drugs and corneal tissues, thus potentially enhancing the intraocular delivery of ophthalmic medications.[6] Among these, mucoadhesive polymers have been reported to be used in order to improve the intraocular penetration of ofloxacin, tobramycin and gentamicin.[6–9]

A novel mucoadhesive polymer consisting of a natural polysaccharide extracted from the medicinal herb *Bletilla striata* and referred to as *Bletilla striata* polysaccharide (BsP) has

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been widely used in East Asian countries to treat alimentary canal mucosal damage, ulcers, bleeding, bruises and burns, and has been investigated for use in the eye.[10,11] Purified BsP is a high molecular weight, non-ionic, neutral, branched polysaccharide.[12] According to test characterization, the monosaccharides in BsP are α-mannose and β-glucose.

The present study was designed to test whether BsP could enhance the transcorneal deposition of antimicrobial drugs, such as levofloxacin, and improve the treatment efficacy of experimental bacterial keratitis when administered topically to healthy rabbits by an ocular drop regimen.

Materials and Methods

Drugs and reagents

D-MEM/F-12 Dulbecco's Modified Eagle Medium was obtained from Invitrogen Corporation (Grand Island, NY, USA). Fetal bovine serum was purchased from Invitrogen Corporation (Grand Island, NY, USA). Levofloxacin and gatifloxacin were a gift from Bausch & Lomb Freda Pharmaceutical Co. (Jinan, China). Ketamine hydrochloride was obtained from Jiangsu Hengrui Medicine Co., Ltd (Lianyungang, China). Chlorpromazine was purchased from Shanghai Harvest Pharmaceutical Co., Ltd, (Shanghai, China). Proparacaine hydrochloride ophthalmic solution was purchased from Alcon, Inc. (Puurs, Belgium). All others chemicals and solvents were of pharmaceutical or chromatographic grade and were used as received unless otherwise specified.

BsP and animals

BsP was purchased from Nanjing Institute for Comprehensive Utilization of Wild Plants (Nanjing, China). The compound had the appearance of small white fibres after freeze drying. It was soluble in water but not in organic solvent. Neither proteins nor nucleic acids were detected in BsP. The BsP contained 98.6% (w/w) total sugar and no uronic acids, and it had a molecular weight of 99 658 Da as indicated by gel filtration chromatography. Glucose and mannose were the only two monosaccharides in BsP, and the molar ratio of mannose and glucose was found to be 1.6 : 1.

Male and female New Zealand albino rabbits were obtained from Shandong Academy of Agricultural Sciences (Jinan, China) (license no: SCXK (Lu) 20040013). Animal care and procedures were conducted according to the Principles of Laboratory Animal Care. The animals were housed individually in an air conditioned and light-controlled room at 25 ± 2°C and at 70 ± 5% relative humidity. They were given a standard pellet feed and provided with water *ad libitum*. All animals were healthy and free of clinically observable ocular abnormalities. The animal study was approved by Shandong Eye Institute Ethics Committee for Animal Experimentation (Qingdao, China).

Cell proliferation assay

Cell proliferation was measured with a MTT assay. The human corneal endothelial cell line (HCECs) (ATCC CRL-11135; kindly donated by Professor Chonn-Ki Joo, The Catholic University of Korea) was used in this study. Briefly, HCECs were grown at 37°C, in a humidified 5% CO_2/95% air atmosphere, in a culture medium of D-MEM/F-12 Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum. Culture medium was replaced every alternate day. Cells were subcultured after 7–10 days (subculture ratio 1 : 5) with 0.25% trypsin containing 0.53 mM EDTA and plated at a density of 250 000 cells/well on 96-well culture plates (Coring Inc., NY, USA). The cell proliferation assay was conducted on HCECs 2 days after seeding. The medium was aspirated and then the cell was incubated in the medium mentioned above without BsP or with different concentrations of BsP (0.0625, 0.125, 0.25, 0.5, 1 or 2%) for 72 h, followed by 4 h incubation with MTT. The MTT transformed crystals were dissolved in DMSO and absorbance at 490 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Experiments were performed in triplicate on six wells for each measurement.

Ferning test

This test was performed by mixing 10.0 μl of 1.0% w/w BsP dispersion with 2.0 μl of artificial lachrymal fluid.[13] The mixture was smeared on a clean microscope slide and allowed to dry at room temperature (25 ± 1°C). The artificial lachrymal fluid had the following composition, given in mg/100 ml of distilled water: $MgCl_2$ 4.75; $CaCl_2$ 7.97; $KHCO_3$ 260.00 and NaCl 754.00. The specimens were examined and photographed with a light microscope equipped with a digital camera (Nikon Eclipse E800; Nikon, Tokyo, Japan).

Drug formulations

The 0.5% levofloxacin-BsP eye drops contained: sterilized water solution containing levofloxacin 5 mg/ml, BsP 10 mg/ml, potassium chloride 3.7 mg/ml, boric acid 1.9 mg/ml, borax 1.9 mg/ml, benzalkonium chloride 0.1 mg/ml and sodium chloride 4.5 mg/ml. The BsP eye drops contained the same components as the 0.5% levofloxacin BsP eye drops except that they lacked levofloxacin. The osmotic pressure of both formulations was measured and ensured to be within the range of 280–330 mOsmol/kg (STY-1E Osmometer; Tianda Tianfa-Technology Co., Ltd., Tianjin, China), and the pH was adjusted to be 6.8–7.2 (Model 828; Orion, Shanghai, China).

Intraocular penetration

A total of 64 New Zealand rabbits (2–2.5 kg) were used for the experiments. The 0.5% levofloxacin eye drops (Cravit) or the corresponding BsP formulations were administered onto the cornea of the unanaesthetised animals using an adjustable micropipette (Eppendorf, Hamburg, Germany), and the eyelids of the animals were gently kept closed for 30 s. Drug solution (50 μl) corresponding to 250 μg levofloxacin was applied. At fixed time intervals after drug administration (5, 10, 15, 30, 45, 60, 120 and 240 min), animals (four rabbits for each formulation and time analysed) were killed by injecting an overdose of ethyl urethane into the marginal ear vein. The aqueous humor was aspirated by anterior chamber paracentesis using a syringe with a 30-gauge needle (BD Ultra-fine Needle Insulin Syringe; Becton Dickinson Company, New Jersey, USA). The aqueous humor samples were stored at −80°C. For analysis, 100 μl of each sample was mixed with 50 μl 10% trichloroacetic acid aqueous solution (w/w) and then centrifuged. The supernatant was then analysed by high performance liquid chromatography

(HPLC). After aqueous humor samples were collected, eyes were rapidly enucleated, trimmed of all adventitial tissue and rinsed with sterile saline. The corneas were then dissected out of the eyes. Following excision, corneas were weighed, suspended (50 mg/ml) in methanol and homogenized as described previously.[6] Samples of corneal tissue were stored at –80°C. For analysis, 500 μl of each sample was mixed with 50 μl of 3 mg/ml gatifloxacin methanol solution, then the mixture was centrifuged and the supernatant was dried under nitrogen flow. The mobile phase (200 μl) was added to the residue and analysed by HPLC.

Treatment of experimental *S. aureus* keratitis

A stock strain of *S. aureus* ATCC25923 (Qingdao Eye Hospital, Qingdao, China) was selected as the test organism. This strain was demonstrated in pilot laboratory tests to induce keratitis comparable with human isolates. The suspension was diluted to 2×10^3 organisms/ml in phosphate buffer solution. Eighteen rabbits were randomly divided into three groups. Anaesthesia was induced by intramuscular injection of 25 mg/kg ketamine hydrochloride and 25 mg/kg chlorpromazine. Topical anaesthesia was achieved by administration of one drop of 0.5% proparacaine hydrochloride to the eye. A 50-μl suspension of *S. aureus* containing 100 organisms was inoculated into the midstroma of the right cornea using a 30 gauge needle. After the intrastromal inoculation, infection was allowed to proceed for 16 h before initiation of antibiotic therapy. Starting from 17 h after inoculation, topical antibiotics were applied to the affected right eye every 15 min for five doses and then every 30 min for 14 doses.[14,15] Each group was treated with one of the following regimens: (i) Cravit (0.5% levofloxacin eye drops); (ii) 0.5% levofloxacin-BsP eye drops; or (iii) 0.9% balanced salt solution as a control.

At 1 h after the final application of the antibiotic drops, the rabbits were killed and uniform corneal buttons were excised with a sterile 8.5-mm trephine (Mingren, Suzhou, China). Corneal buttons were ground in 1 ml sterile phosphate buffer solution using a disposable tissue homogenizer (Model 985370, Tissue-Tearoe; BioSpec Products, Inc., Bartlesville, OK, USA). Serial dilutions were then prepared in sterile phosphate buffer solution. Samples (20 μl) from each dilution were plated on agar plates and incubated at 35°C. Quantitative analysis of viable *S. aureus* colonies was conducted on the Day 3 of incubation.

Quantitative determination of levofloxacin

Quantitative determination of levofloxacin was performed on a HPLC system comprising a G1314A UV detector (detection at a maximum of 294 nm; Agilent, Palo Alto, California, USA), G1367A injector and G1311A Quat Pump (Agilent, Palo Alto, California, USA). Reversed phase columns Agilent ZORBAX SB-C18 (250 mm × 4.60 mm, 5 μm; Agilent, Palo Alto, California, USA) were used for separations. Gatifloxacin was used as an internal standard. The eluent for the levofloxacin consisted of 19% acetonitrile and 81% 0.02 mol/l phosphate buffer (containing 0.1% triethylamine, with pH adjusted to 4.0 using 85% phosphoric acid). The flow rate was kept constant at 1.0 ml/min. The detection was performed at 40°C. The retention time of levofloxacin and gatifloxacin was 4.7 and 7.9 min, respectively. The limit of determination of levofloxacin was 53.95 ng/ml.

Ocular irritation testing

Rabbits were divided into two groups (six rabbits per group) and then treated 6 times a day (one drop in the right eye) with different formulations for 2 weeks. The left eyes served as controls and were treated with saline. Group 1 was treated with the 1% BsP eye drops and group 2 with 0.5% levofloxacin-BsP eye drops. The ocular condition was recorded every day until 1 h after the last administration. According to the Draize test, ocular irritation scores for every rabbit were calculated by adding together the irritation scores for the cornea, iris and conjunctiva.[16] The eye irritation score was obtained by dividing the total scores for all rabbits by the number of rabbits.

Statistical analysis

The intraocular penetration results were expressed as mean ± SD. Statistical analysis was performed using the two-tailed Student's *t*-test. Pharmacokinetic analysis was carried out by fitting mean drug concentration versus time data. Pharmacokinetic parameters were obtained using the practical pharmacokinetic program DAS2.0 (Mathematic Pharmacology Professional Committee of China, Shanghai, China). For quantitative analysis of the efficacy of the experimental keratitis treatment, data were transformed to logarithmic values and one-way analysis of variance was used to compare the efficacy of each regimen. $P < 0.05$ was considered statistically significant.

Results

Cell proliferation assay

A cell proliferation assay was carried out to examine the toxicity of different concentrations of BsP. The results indicated that BsP did not inhibit cell growth (Figure 1). After addition of BsP at concentrations ranging from 0.0625 to 1%, the proliferative efficacy of HCECs was increased compared with the control group and this increase was directly dependent on the concentration. When the concentration was increased to 2%, the proliferative efficacy was decreased but HCECs were still stimulated compared with the control group. This could be explained by the fact that BsP is a mucoadhesive polymer, and

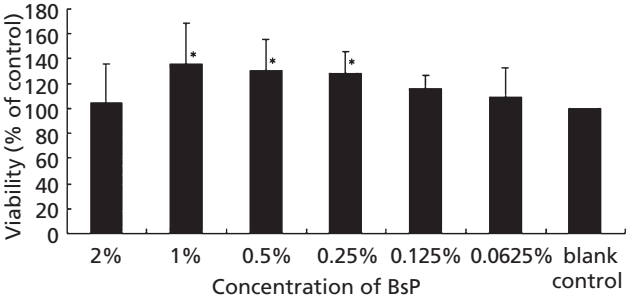

Figure 1 Cell proliferation assay. BsP, *Bletilla striata* polysaccharide; *$P < 0.05$, significantly different compared with the blank control ($n = 3$).

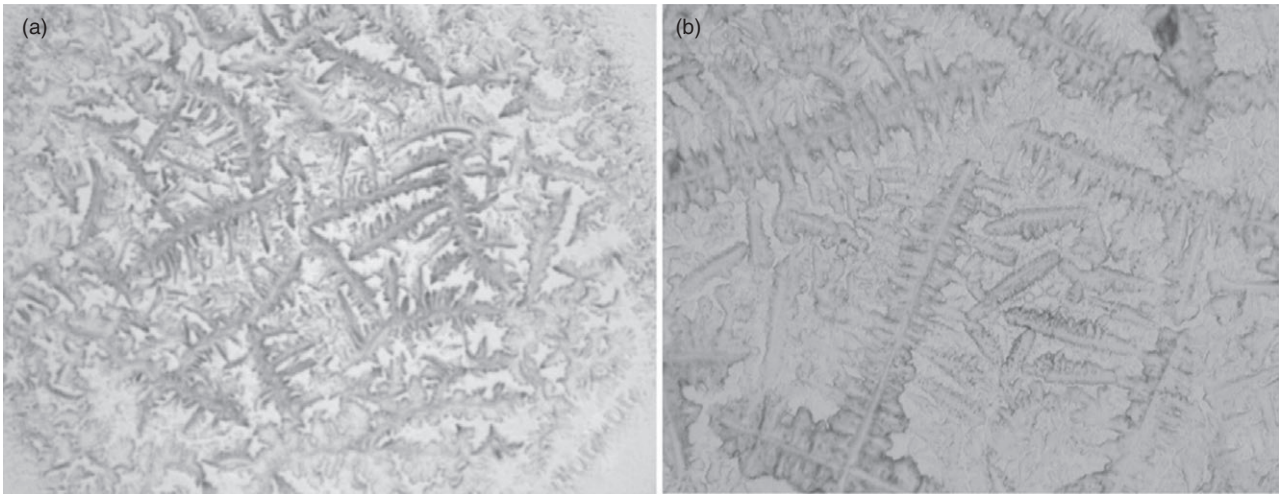

Figure 2 Ferning test. (a) 1.0% *Bletilla striata* polysaccharide dispersion. (b) Human lachrymal fluid. Magnification 100×.

the viscosity was too high at a concentration of 2%, thus decreasing the proliferative efficacy.

A 1% BsP solution was most effective. The viscosity of the 1% BsP dispersion was 13.9 mPa s (determined using a NDJ-7 rotating cylinder viscometer; Shanghai Precision Instrumentation Co., Ltd, Shanghai, China), with shear rates of 750 r/min and temperature of 25 ± 0.2°C; this viscosity was similar to some other ophthalmic formulations. The ideal viscosity of ophthalmic solutions has been shown to be over the range of 4–5 mPa s,[17] and the maximum allowable viscosity should be 15–30 mPa s.[18] Thus, we used 1% BsP in the following studies.

Ferning test characteristics of the BsP dispersion

The ferning test showed that BsP crystallized into type I fern-like structures similar to those produced by human lachrymal fluid (Figure 2). This study showed that polymers with the best muco-mimetic properties would crystallize into fern-like patterns analogous to those produced by lachrymal mucus. Such properties were seen in BsP solutions. Thus, we conclude that BsP exhibited optimal performance as a tear fluid.

Intraocular penetration

The levofloxacin concentrations in the 5-min aqueous humor samples of both levofloxacin eye drops were below the detection limit. The concentration–time curve of levofloxacin in the aqueous humor is shown in Figure 3. At all time points, the levofloxacin concentration sequence was 0.5% levofloxacin-BsP eye drops > Cravit, and the C_{max} values for the two conditions were significantly different ($P < 0.05$). The $AUC_{0\to t}$ was 230.137 mg/l min and 128.314 mg/l min for 0.5% levofloxacin-BsP eye drops and Cravit, respectively. The relative bioavailability of 0.5% levofloxacin-BsP eye drops was 179.35% compared with Cravit. Thus, we conclude that BsP significantly increased the aqueous humor bioavailability of levofloxacin. The pharmacokinetic parameters are given in Table 1. Pharmacokinetic fitting results showed that the 0.5% levofloxacin-BsP eye drops and Cravit belonged to a two-compartment model with a weight coefficient of 1/cc. The cornea and other periocular tissues could be fitted as the first compartment and the aqueous humor as the second compartment.

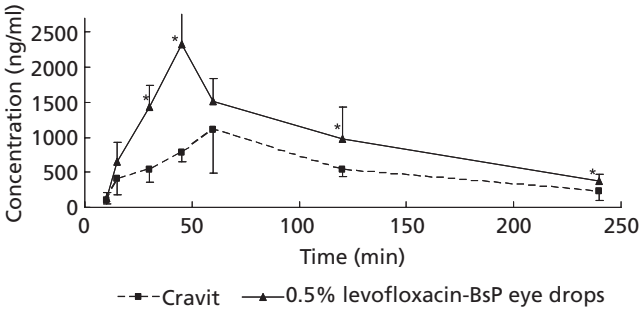

Figure 3 Levofloxacin concentration–time profile in the aqueous humor after treatment with 0.5% levofloxacin-BsP eye drops or Cravit. BsP, *Bletilla striata* polysaccharide; Cravit, 0.5% levofloxacin eye drops. *$P < 0.05$, significantly different compared with Cravit ($n = 8$).

Table 1 Pharmacokinetic parameters in the aqueous humor

Parameter	0.5% Levofloxacin-BsP eye drops	Cravit
$t_{1/2\beta}$ (min)	90.512	106.48
V_1 (l)	0.235	0.117
Cl (l/min)	0.002	0.001
t_{max} (min)	45	60
C_{max} (mg/l)	2.319	1.111
AUC_{0-t} (mg min/l)	230.137	128.314
$AUC_{0-\infty}$ (mg min/l)	280.076	163.720

BsP, *Bletilla striata* polysaccharide; Cravit, 0.5% levofloxacin eye drops; $t_{1/2\beta}$, elimination half life; V_1, apparent volume in the central compartment; Cl, clearance; t_{max}, time to reach maximum concentration; C_{max}, maximum concentration; AUC_{0-t}, area under the concentration–time curve from time zero to time t; $AUC_{0-\infty}$, area under the plasma concentration–time curve from time zero to infinity.

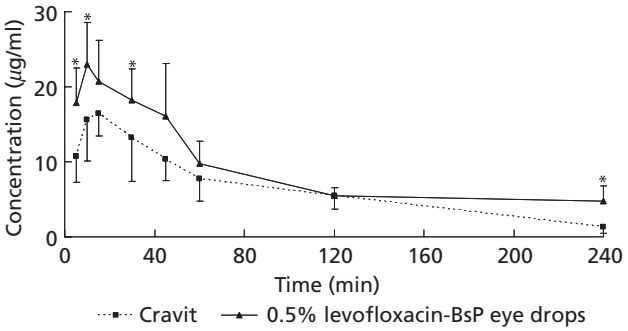

Figure 4 Levofloxacin concentration–time profile in the cornea after treatment with 0.5% levofloxacin-BsP eye drops or Cravit. BsP, *Bletilla striata* polysaccharide; Cravit, 0.5% levofloxacin eye drops. *$P < 0.05$, significantly different compared with Cravit ($n = 8$).

Table 2 Pharmacokinetic parameters in the cornea

Parameter	0.5% Levofloxacin-BsP eye drops	Cravit
$t_{1/2\beta}$ (min)	109.4	64.956
V_1 (g)	14.022	14.318
Cl (g/min)	0.089	0.153
t_{max} (min)	10	15
C_{max} (μg/g)	23.012	16.389
AUC_{0-t} (μg min/g)	2066.764	1510.544
$AUC_{0-\infty}$ (μg min/g)	2814.605	1636.649

BsP, *Bletilla striata* polysaccharide; Cravit, 0.5% levofloxacin eye drops; $t_{1/2\beta}$, elimination half life; V_1, apparent volume in the central compartment; Cl, clearance; t_{max}, time to reach maximum concentration; C_{max}, maximum concentration; AUC_{0-t}, area under the concentration–time curve from time zero to time t; $AUC_{0-\infty}$, area under the plasma concentration–time curve from time zero to infinity.

The concentration–time curve of levofloxacin in cornea samples is shown in Figure 4. Similar to aqueous humor samples, the levofloxacin concentration sequence was 0.5% levofloxacin-BsP eye drops > Cravit at all time points, and their C_{max} values were significantly different ($P < 0.05$). The $AUC_{0\rightarrow t}$ was 2066.764 and 1510.544 μg min/g for 0.5% levofloxacin-BsP eye drops and Cravit, respectively. The relative corneal bioavailability of 0.5% levofloxacin-BsP eye drops was 136.82% compared with Cravit. Thus, we conclude that BsP increased the corneal accumulation of levofloxacin.

The pharmacokinetic parameters are given in Table 2. Curves fitted to the pharmacokinetics showed that the 0.5% levofloxacin-BsP eye drops and Cravit belonged to a two-compartment model, with a weight coefficient of 1/cc. The lacrimal fluid and lacrimal film could be fitted as the first compartment and the cornea as the second compartment.

Treatment of *S. aureus* keratitis

At 16 h after inoculation, *S. aureus* keratitis developed in all 18 rabbits. Corneal infiltrates appeared as focal, fluffy white deposits with fine radiating projections, similar to observed and reported clinical signs in culture-proven cases of *S. aureus* keratitis in humans.

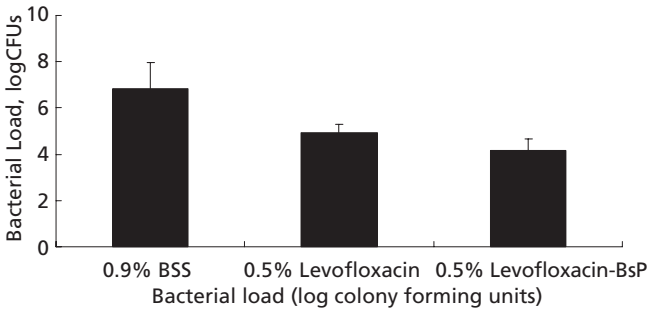

Figure 5 Results of treatment of *Staphylococcus aureus* keratitis. BSS, topical balanced salt solution; BsP, *Bletilla striata* polysaccharide. ($n = 6$).

The quantitative culture results are shown in Figure 5. Treatment of the infected eyes with 0.5% levofloxacin and 0.5% levofloxacin-BsP eye drops led to 4.94 ± 0.33 *S. aureus* organisms and 4.17 ± 0.46 log colony-forming units/cornea, while those treated with 0.9% balanced salt solution had values of 6.85 ± 1.13. The 0.5% levofloxacin and 0.5% levofloxacin-BsP eye drops significantly reduced the number of *S. aureus* compared with the untreated control group (both $P < 0.05$), and levofloxacin-BsP therapy demonstrated greater antibacterial activity than levofloxacin therapy ($P < 0.05$).

Ocular irritation

To exclude the effect of osmotic pressure, each formulation was measured and found to be equivalent to normal saline. During the ocular irritation tests, no ocular damage or clinically abnormal signs were observed in the cornea, conjunctiva or iris. The irritation score for both groups was less than 1, indicating that both formulations had excellent ocular tolerance.

Discussion

B. striata has been used medicinally for over 1500 years in traditional Chinese medicine.[10] Its root has antibacterial, anti-inflammatory and demulcent properties.[10,19] There are no reports of *B. striata* causing adverse reactions in its long history of use as a medicinal herb. BsP has been widely used in the clinic, including external and internal application, and arterial embolization,[20] and there have been no reports of adverse reactions or toxicity. Although there was no further evidence, our cell cytotoxicity and ocular irritation tests indicated that BsP was safe for ophthalmic drug delivery.

BsP is not an inert material and it has been used and investigated widely in recent years. Wang *et al*. showed that BsP could induce the proliferation of human umbilical vascular endothelial cells and the expression of vascular endothelial growth factor up to 156 and 147% of control values.[21] In our study, the cell proliferation results showed that BsP can significantly induce the proliferation of HCECs. These findings suggest that BsP may reduce the toxicity of some drugs on the cornea. This is an important characteristic as previous studies have shown that many antimicrobial drugs, such as ofloxacin and ciprofloxacin, cause a clear alteration of reepithelialization in the wounded eye.[22,23]

An interesting physical characteristic of tears is that when they are allowed to dry on a microscope slide at room temperature, they crystallize into fern-like structures. As described by Burgalassi *et al*., tear-film crystallization (i.e. ferning or arborization) is classified into four types of structures according to their appearance.[13] The ferning test was used to evaluate polymeric artificial tear substitutes. The ferning test evaluation showed that BsP solutions had the best muco-mimetic properties, analogous to those produced by lachrymal mucus itself. Such properties indicated that BsP would have good biocompatibility with lacrimal fluid.

In the intraocular penetration study, the significant increase in drug concentration obtained in aqueous humor and corneal samples by using BsP as a delivery system suggests that this natural polysaccharide enhances intraocular absorption and corneal drug accumulation. A similar effect has been observed with other polysaccharides such as chitosan and tamarind-gum polysaccharide.[8,9] In the treatment of *S. aureus* keratitis, levofloxacin-BsP reduced *S. aureus* in the cornea at a higher rate than levofloxacin alone. These results indicate that levofloxacin bioavailability in the cornea is increased by BsP, which is in accordance with the intraocular penetration results. Therefore, it may be assumed that other active antibiotics can also reach higher intracorneal concentrations and be more effective in controlling bacterial replication in the cornea when delivered using polysaccharides.

Conclusions

The results of this investigation suggest that BsP can be successfully used as an ophthalmic delivery system for antimicrobial drugs. Its use leads to an increase in the aqueous humor and corneal bioavailability of levofloxacin and to improved treatment efficacy for experimental bacterial keratitis. The use of BsP may also be possible for other medications used for topical treatment of eye disease. Further research is needed to develop new drug formulations with BsP in order to promote its use.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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