Original article

The antibacterial activity of levofloxacin eye drops against staphylococci using an *in vitro* pharmacokinetic model in the bulbar conjunctiva

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ABSTRACT

The incidence of fluoroquinolone-resistant staphylococcal isolates from the conjunctival sac is increasing. We compared pharmacological effects of levofloxacin (LVFX) against *Staphylococcus epidermidis* using an *in vitro* pharmacokinetic (PK) model simulating the concentration in the bulbar conjunctiva after applying eye drops of 0.5% and 1.5% LVFX. We used *S. epidermidis* conjunctival sac isolates [minimum inhibitory concentrations (MICs) of LVFX, 0.125 μg/mL]. LVFX-resistant strains were obtained from parental strains after culture with LVFX. The *in vitro* PK model simulated the concentration in the bulbar conjunctiva following three topical applications of 0.5% or 1.5% LVFX ophthalmic solution (0, 4, and 8 h) to rabbit eyes. Parental and LVFX-resistant strains were exposed to LVFX in the *in vitro* PK model, and changes in viable bacterial counts were evaluated for 12 h. The MICs of LVFX for the resistant isolates were 2–32 times higher than the parental strain, and those with MICs ≥2 ug/mL had mutations in the quinolone resistance-determining region. The PK model simulation predicts that 1.5% LVFX exerts bactericidal and bacteriostatic effects against strains with MICs of 0.125–2 and 4 μg/mL, respectively, whereas 0.5% LVFX would only be effective against strains with MICs of 0.125–1 μg/mL. The PK model predicts that the 1.5% LVFX ophthalmic solution exhibits a stronger bactericidal effect against resistant staphylococci in the bulbar conjunctiva than the 0.5% LVFX ophthalmic solution.

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1. Introduction

Antibiotic eye drops provide an effective treatment of ocular infections such as keratitis and conjunctivitis. In contrast to systemic antibiotics, eye drops rapidly deliver high concentrations of antibiotics at the site of infection. The antibiotics available in eye drop preparations include fluoroquinolones, aminoglycosides, cephalosporins, oxytetracycline, and macrolides. Because of their high stability, broad spectrum, and efficacy, fluoroquinolone eye drops are increasingly used for the treatment of bacterial keratitis and conjunctivitis by general practitioners in many countries [1–3].

Antibiotic eye drops are also used for the prevention of postoperative ocular infections [4–7]. Infectious endophthalmitis is a rare but serious complication of cataract surgery, which may lead to the loss of visual acuity [8]. The bacterial flora found in the external eye is predominantly responsible for postoperative endophthalmitis, particularly by gram-positive cocci such as *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), and *Enterococcus faecalis* [9,10]. Many of these strains were detected in conjunctival sac of patients scheduled to undergo cataract surgery [7]. Thus, the elimination of these gram-positive cocci from the conjunctival sac is important to prevent postoperative endophthalmitis.

Pharmacokinetics and pharmacodynamics (PK/PD) is an important concept when considering the use of antibiotics. Based on these analyses, a single daily systemic administration of high-dose fluoroquinolone is recommended because of their concentration-dependent antibacterial activity and long half-lives [11]. *In vitro* simulation systems are widely used to understand the PK/PD of antibiotics [12].

Antibacterial ophthalmic solutions containing fluoroquinolones are widely used for perioperative disinfection because they effectively eradicate bacteria before eye surgery [4–7]. However, there is an increasing incidence of fluoroquinolone-resistant bacteria in the external eye due to the development of drug-resistant CNS after the

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application of topical fluoroquinolones [13—15]. Thus, the possible lower effectiveness of topical fluoroquinolones for perioperative eradication is a major concern for ophthalmologists. In Japan, several types of fluoroquinolone eye drop solutions are available in the market. In particular, levofloxacin (LVFX) eye drops are used at two concentrations: 0.5% and 1.5%. Although 1.5% LVFX was proved to be effective in eradicating bacteria from the conjunctival sac before surgery [6], no study compared the efficacy of 0.5% and 1.5% LVFX. Furthermore, only few PK/PD studies targeted antibiotic eye drops. In the present study, we developed an *in vitro* PK model to simulate the antibiotic concentration in the bulbar conjunctiva following the application of 0.5% or 1.5% LVFX eye drops, compared their bactericidal effects against *Staphylococcus epidermidis* strains with variable susceptibility, and checked if these eye drops were effective to kill fluoroquinolone-resistant bacteria.

2. Materials and methods

2.1. Bacteria and reagents

Clinical strains of *S. aureus* and *S. epidermidis* with variable susceptibility to LVFX were isolated from the conjunctival sac before cataract surgery (Table 1). The bacteria were cultured overnight in brain heart infusion broth (Becton Dickinson and Co., Sparks, MD, USA) at 37 °C and then suspended in sterile saline to obtain a suitable concentration for inoculation. LVFX was obtained from Daiichi-Sankyo Co. Ltd. (Tokyo, Japan).

2.2. Animal care and use

Male white rabbits were obtained from Kitayama labes Co. Ltd. (Nagano, Japan). All animals were housed and treated according to the guidelines of the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

2.3. Induction of LVFX-resistant strains

We used the *S. epidermidis* (E1) strain, for which the MIC of LVFX was 0.125 μg/mL. The LVFX-resistant strains were developed from the E1 strain as described previously [16]. In brief, the E1 strain was cultured overnight in Mueller—Hinton broth (MHB) and

concentrated by centrifugation to yield 10^9—10^{10} CFU/mL as the final inoculum. This suspension was spread onto Mueller—Hinton agar (MHA). After a 48 h incubation at 37 °C, the colonies with low sensitivity for LVFX were picked and cultured in MHB to measure the MICs. The most resistant strains with high MICs were used for a second round of LVFX resistance induction to obtain the LVFX-resistant strains used in the present study.

2.4. Amplification and sequencing of quinolone resistance-determining regions (QRDRs)

The clinical strains and LVFX-resistant strains were analyzed to obtain the mutation profiles of the QRDRs in the A subunits of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*). The amplification of the QRDRs in *gyrA* and *parC* was performed as described previously [17]. In this study, the mutation in *gyrB* and *parE* was not examined because the QRDRs in *gyrA* and *parC* was predominantly responsible for fluoroquinolone resistance.

2.5. Concentration-time profiles of LVFX in the bulbar conjunctiva

A single dose of ophthalmic solution containing 0.5% or 1.5% LVFX was instilled into the eyes of each white rabbit. The rabbits were allowed to move freely in their cage until euthanasia was performed for sample collection. After 0.25, 0.5, 1, 2, 4, 6, and 8 h, the animals were euthanized by excessive sodium pentobarbital injection (n = 5—6). The bulbar conjunctiva were collected, weighed, and homogenized with 1 mL of 1% acetic acid/methanol mixture (30:70) and zirconia beads in a Shake Master Auto (Biomedical Science, Tokyo, Japan). A supernatant was obtained from each sample by centrifugation. Then, a 50 μL aliquot was mixed with 5 μL of 0.2% acetic acid, followed by 200 μL of 250 ng/mL lomefloxacin in a water/acetonitrile mixture (10:90). Each sample was applied to a Strata Impact Protein Precipitation plate (Phenomenex, CA, USA) and centrifuged. After removal of solvent from eluent, each sample was reconstituted by 75 μL of mobile phase and injected into the UPLC system for determination of LIVX concentration. The HPLC system (AQUITY® UPLC system, Waters) with AQUITY® BEH C18 column (1.7 μm,. 100 mm length × 2.1 mm i.d., Waters), was used for the assay. A mixture of 0.005 mol/L potassium phosphate buffer (pH 3.0) and acetonitrile (90/10, v/v) was used as the mobile phase with a flow rate of 0.6 ml/min. Retention of the drug was monitored with a fluorescence spectrophotometric detector (Ex. 294 nm, Em. 510 nm).The values expressed in μg/g of tissue were converted to μg/mL.

2.6. In vitro PK model

An *in vitro* PK model based on agar chips was developed to simulate the antibiotic concentration in the bulbar conjunctiva following the topical application of 0.5% or 1.5% LVFX eye drops in rabbits. The LVFX concentration in the culture medium was adjusted to the concentration-time profiles in the bulbar conjunctiva after three instillations were performed every 4 h for 12 h. In brief, overnight cultures of the test strains were mixed with 1% liquefied MHA in a 55 °C water bath and were adjusted to 10^8 CFU/mL. Then, 200 μL of liquefied MHA containing bacteria was dropped onto a sterile plastic plate and allowed to solidify. The agar chips were subjected to stepwise *in vitro* PK analysis. The agar chips were gently rinsed with sterile MHA and transferred to fresh culture medium containing different LVFX concentrations. The agar chip of control was transferred to medium without LVFX. The agar chips were collected after 0, 4, 8, and 12 h and homogenized with glass beads. The homogenized chips were used to determine bacterial

Table 1
Impact of gene mutations on bacterial susceptibility to LVFX.

Strain	Organism	MIC μg/mL	Mutation in QRDR	
			gyrA	*parC*
A1	*Staphylococcus aureus*	0.25	—	—
E1	*S. epidermidis*	0.125	—	—
E2	*S. epidermidis*	0.25	—	—
E3	*S. epidermidis*	0.25	—	—
E4	*S. epidermidis*	0.25	Glu208 → Lys	—
E5	*S. epidermidis*	0.25	Asp142 → Asn	—
E6	*S. epidermidis*	2	Ser84 → Phe	Ser80 → Phe
E7	*S. epidermidis*	2	—	Ser80 → Phe
E8	*S. epidermidis*	4	—	Ser80 → Phe
E9	*S. epidermidis*	4	Ser84 → Phe	Ser80 → Tyr
E10	*S. epidermidis*	4	Ser84 → Phe	Ser80 → Tyr
E11	*S. epidermidis*	4	Ser84 → Phe	Ser80 → Tyr
E12	*S. epidermidis*	4	Ser84 → Phe	Ser80 → Tyr
E1-R1	*S. epidermidis*	0.5	—	—
E1-R2	*S. epidermidis*	1	—	—
E1-R3	*S. epidermidis*	2	Ser84 → Tyr	—
E1-R4	*S. epidermidis*	4	Ser84 → Tyr	—
E1-R5	*S. epidermidis*	4	Ser84 → Tyr	His103 → Tyr

MIC, minimum inhibitory concentration; QRDR, quinolone resistance-determining region.