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Development of topical chitosan/ β -glycerophosphate-based hydrogel loaded with levofloxacin in the treatment of keratitis: An *ex-vivo* study

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ABSTRACT

Staphylococcus species are responsible for most cases of post-operative endophthalmitis. Topical ocular drug was applied for post-operative infection prevention, but the way of delivery encounters many challenges in terms of patient's compliance, drug efficacy, and drug penetration. We used the levofloxacin-loaded chitosan/gelatin/ β -glycerophosphate hydrogel sustained releasing system with good *in vitro* anti-bacterial efficacy and biocompatibility, which we had previously designed, for *ex vivo* keratitis model to test the preclinical drug efficacy and to determine drug level in the anterior chamber of the eye. The result showed that the *ex-vivo* corneal keratitis model with *S. aureus* infection revealed mild opacity over the central cornea with stromal infiltrate, but without obvious stromal infiltration post levofloxacin-loaded hydrogel treatment after 24 h of infection. Quantification of viable bacteria showed a significant anti-bacterial activity. The histological evidence also showed no visible *S. aureus* after levofloxacin-loaded hydrogel treatment, with a significant anti-inflammatory effect. We also examined the drug concentration in the aqueous humor 24 h after instilling one drop of the levofloxacin-loaded hydrogel. The concentration achieved to a desired drug level. These results suggested that by the *ex-vivo* model, levofloxacin-loaded hydrogel can be applied for treatment in post-operative endophthalmitis or keratitis after the ophthalmic surgery.

1. Introduction

Cataract surgery is one of the most frequently performed ocular procedures in the world [1]. The incidence of post-operative endophthalmitis (POE) following cataract surgery varies from country to country. Based on studies that analyzed general population data, the reported incidence of POE ranges from 0.05% to 0.68% [2, 3, 4, 5, 6]. Although the incidence rate is low, a large number of patients receive cataract surgery every year worldwide. Therefore, this dreadful and sight-threatening event may become a large socioeconomic burden [7, 8, 9, 10, 11].

Bacteria, mainly *Staphylococcus* species [6, 7, 8, 9, 10, 11], are responsible for most cases of POE [12, 13, 14, 15, 16]. Fluoroquinolones have been recommended as the preferred option for preventing ocular infections [17]. Several maneuvers have been adopted to reduce the risk

of developing POE, including the use of topical preoperative antibiotics, a topical application of a povidone-iodine combination to the ocular surface before surgery, an instillation of antibiotics into the infusion bottle during surgery, an intracameral injection of antibiotics at the end of surgery, and a postoperative subconjunctival or topical application of antibiotics [18, 19, 20, 21]. Levofloxacin, a hydrophobic fluoroquinolone, is routinely applied four times per day for at least 14 days after cataract surgery [22, 23]. However, topical ocular drug delivery presents many challenges, including patient compliance and drug efficacy. Patient compliance is challenged by the difficult instillation technique, the necessary timing, and the frequency of administering drops in the eye [24, 25]. Drug efficacy is challenged by the induced lacrimation, tear turnover, blinking, and tear dilution, which can reduce the ocular bioavailability of the drug. Moreover, the relative impermeability of the

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cornea leads to less than 5% of drug penetration into the intraocular area [26, 27].

A thermosensitive chitosan/β-glycerophosphate-based drug delivery system, which forms the hydrogel without a chemical reaction, has been widely used as a sustained drug-releasing system in research for ophthalmic diseases. It changes phase with variation in temperature; being aqueous solution at low temperatures and undergoes volume phase transition to become gels when the temperature exceeds the lowest critical temperature. The advantages of the hydrogel lie in its excellent viscosity at gel form, and thermal sensitivity as it transforms rapidly at critical temperature. Several in situ-forming hydrogels have been designed as topical eyedrops for application in the field of ophthalmology. As the hydrogel eyedrops instilled and contact the ocular surface, it undergoes immediate gelation, and release the drug packed inside slowly [28, 29, 30, 31]. The chitosan/β-glycerophosphate-based hydrogel can incorporate hydrophilic or hydrophobic therapeutic molecules by directly mixing at room temperature [32, 33]. The tensile strength of the chitosan/β-glycerophosphate hydrogel can be augmented by adding a second polymer, such as gelatin, collagen, or dextran [34, 35, 36].

Chitosan is a natural polysaccharide with mucoadhesive properties that may increase the precorneal retention time when applied topically [37]. Gelatin is a water-soluble, natural polymer. The addition of gelatin to a chitosan/ β -glycerophosphate hydrogel can reduce the gelation temperature (to ~34 °C) and prolong the release of a hydrophobic drug for weeks to months [38]. There have been many studies using different hydrogels as platforms for topical ophthalmic antibiotics and antifungal agents to treat keratitis [39, 40, 41, 42, 43, 44]. However, none were applicated with chitosan/gelatin/ β -glycerophosphate hydrogel. In our previous *in vitro* study [45], we have developed a levofloxacin-loaded chitosan/gelatin/ β -glycerophosphate hydrogel that increases the retention time of drugs on the ocular surface, which is important for maintaining an effective therapeutic drug concentration. It was optimized no matter in temperature for sol-gel transition on ocular surface, or osmolarity suitable for topical use [46].

In the present study, we created an *ex vivo* keratitis model to test the efficacy of our levofloxacin-loaded, chitosan-based sustained drug release system. Based on the method that Pinnock et al described [47], we modified the way of creating area of infection and the way of causing bacterial infection. To the best of our knowledge, this modified method has not been used in the previous studies. This *ex vivo* keratitis model served as a preclinical surrogate to verify the penetration of the drug into the anterior chamber of the eye and to test the effectiveness of the newly developed levofloxacin-loaded hydrogel in preventing the development of POE.

2. Materials and methods

2.1. Preparation of the thermosensitive levofloxacin-loaded hydrogel

The chitosan-gelatin based levofloxacin-loaded hydrogels were prepared as described previously [45]. In brief, we first prepared the chitosan/gelatin/β-glycerophosphate solution. Chitosan (2%, Xing Cheng Biochemical Factory Nantong, China) and gelatin (0.2%, G2500, Sigma, USA) were dissolved in 0.1 M acetic acid (242853, Sigma, USA), then sterilized in the autoclave. The chitosan was >95% deacetylated, with a viscosity of 581 mPa s. We prepared a glycerol 2-phosphate disodium salt hydrate (GP) solution by dissolving 20 g of GP (G5422, Sigma, USA) in 25 ml of double distilled H₂O. Then, we then sterilized the solution by passing it through a 0.22 µm filter (Millex-GV, Millipore, USA). Next, the GP solution was added, drop-by-drop, to the chitosan/gelatin solution with continuous stirring, until the pH was 7.4. This product was stored at 4 °C. For the final step, the chitosan/gelatin/ β -glycerophosphate solution was placed in an ice-water bath under a laminar flow hood, and levofloxacin (28266, Sigma, USA) was added while stirring, until the concentration reached 10 mg/ml in the hydrogel. This final product was stored at 4 °C until further use. The gelation properties of levofloxacin (10 mg/mL) loaded thermosensitive chitosan-based hydrogel were analyzed using a rheometer. The gelation temperature of levofloxacin-containing hydrogel was 34.15 °C \pm 0.17 °C. The osmolality of developed hydrogel was evaluated by extraction method. The samples were measured by the Advanced Osmometer (Model 3250, Advanced Instruments). The results showed that the osmolality of the developed hydrogel was 304.0 \pm 4.1 mOsm/L. The biocompatibility of the developed hydrogel on RCE cells, as well as gelation temperature and osmolarity have been performed in our previous study [45]. The drug release of levofloxacin *in vitro* displayed a sustained-release profile.

2.2. Bacterial culture

Staphylococcus aureus bacteria (American Type Culture Collection 25923) were cultured in Mueller-Hinton broth. For experiments, bacterial solutions were grown to a turbidity of 0.5 McFarland standard ($\sim 1 \times 10^8$ colony forming units [CFU]/ml).

2.3. Ex-vivo rabbit corneal organ culture

The animal experiment was approved by the Ethics Committee for Animal Research of the Taipei Veterans General Hospital. New Zealand albino rabbits (body weight ~ 2 kg) were maintained and treated in accordance with the guidelines for the care and use of laboratory animals. After euthanasia, the corneas with sclera rims were dissected with a standard procedure, including decontamination with povidone-iodine, and they were immediately placed into phosphate buffered saline (PBS). Ex vivo rabbit corneal organ cultures were established as previously described [31]. Briefly, rabbit corneoscleral buttons were placed in culture dishes, epithelial side down, and 500 µl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5% (w/v) agarose was added to the endothelial side of the cornea. Corneas were then placed epithelium-side up, and we added DMEM/F12, supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2.5 µg/ml amphotericin B, 5 µg/ml insulin, and 10 ng/ml epidermal growth factor, until the corneas were submerged.

2.4. Ex vivo rabbit model of S. aureus keratitis

We prepared the ex vivo keratitis model based on the method described by Pinnock et al [40]. Briefly, corneas were washed with PBS and cultured in 12-well plates containing antibiotic-free medium for 24 h. After washing with PBS, the samples were randomly divided into three groups. The first group comprised intact corneas (control). The second group comprised infected corneas (infected group). In this group, corneas were wounded with a metal ring (3mm in diameter) and a scalpel to create an epithelial defect with a 3-mm diameter. Then, immediately after wounding the cornea with scalpel, the bacterial suspension was added, and the plates were incubated for 24 h at 37 °C. The third group comprised infected corneas that were treated with drug-loaded hydrogels (treated group). In this group, the corneas were wounded, and the bacterial suspension was added immediately, as described for the infected group. Then, the infected corneas were immediately treated with 50 µl of levofloxacin-loaded thermosensitive hydrogel. The samples were incubated for 24 h at 37 °C.

2.5. S. aureus bacterial quantification in infected corneal tissues

The control, infected, and treated groups of corneas were homogenized separately and cultured to evaluate bacterial growth. Briefly, samples were homogenized and diluted with PBS. The sample homogenates were placed into culture tubes containing Bacto[™] Tryptic Soy Broth, and the tubes were incubated for 24 h at 37 °C. Next, cultures were centrifuged, and the supernatant of each sample was collected and added to a dish containing agar medium. The dishes were incubated at 37 °C for

Y.-F. Chang et al.

24 h. The CFUs in each sample were quantified at 600 nm with a spectrophotometer.

2.6. RNA extraction and quantification of inflammatory gene expression

The control, infected, and treated groups of corneas were homogenized, and total RNA was extracted with TRIzol® Reagent, according to the manufacturer's instructions. The total RNA yields were quantified with the NanoVue Plus spectrophotometer (GE) at 260 and 280 nm. The 260/280-nm ratios were between 1.8 and 2.0. RNA was suspended in DEPC-treated water and stored at -80 °C for the real-time reverse transcription-polymerase chain reaction (RT-PCR) assay.

Next, cDNA was synthesized from RNA with the SuperScriptTM III First-Strand Synthesis System for RT-PCR. Quantitative PCR was performed with the power SYBR green PCR master mix, according to the manufacturer's instructions. Reactions were performed with a StepOneTM Fast real-time PCR System. We examined expression of the following target genes: tumor necrosis factor- α (*TNF-\alpha*), interleukin-1- α (*IL-1\alpha*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), matrix metalloproteinase-3 (*MMP-3*), and metalloproteinase-9 (*MMP-9*). Glyceraldehyde-3phosphate dehydrogenase mRNA served as the endogenous housekeeping gene for all experiments. The relative expression of each target gene was examined with the 2^{- $\triangle \triangle Ct$} method.

2.7. Histological analysis

The control, infected, and treated groups of corneal tissues were fixed in 10% formalin (HT501128, Sigma, USA) for 24 h. Then, the samples were dehydrated in a graded ethanol series and embedded in paraffin blocks. Next, the samples were cut into 5-µm-thick sections and stained with hematoxylin and eosin (H&E; Muto Pure Chemicals, Japan) and being analyzed under the microscope.

2.8. Aqueous humor drug concentrations

To determine the drug concentration, three animals were anesthetized with 10 mg/kg Zoletil 50 and topical anesthesia (0.5% proparacaine hydrochloride). Next, one drop of thermosensitive levofloxacinloaded hydrogel was applied to the ocular surface. Then, after 1, 3, 6, 24, and 48 h, 100 μ l of aqueous humor was collected with a 30-gauge needle attached to a 1 cc syringe. These samples were added to 300 μ l acetonitrile and stored at 4 °C. The samples were analyzed with a liquid chromatography in tandem with mass spectrometry (LC-MS/MS) system, equipped with an electrospray ionization source. The LC-MS/MS system included an 1100 series LC system (Agilent) and an API 3000 tandem mass spectrometer (Sciex).

2.9. Statistical analysis

Data are reported as the mean \pm standard error of the mean, for at least three replicate experiments. Statistical comparisons were performed with the Student's t test and the one-way analysis of variance (ANOVA), followed by Turkey's test. P-values <0.05 were considered statistically significant.

3. Results

3.1. External photography of dissected rabbit cornea with scleral rim

We acquired photographs of the rabbit corneas at baseline, and at 6 and 24 h after treatments. In the control group, the rabbit corneas exhibited intact epithelium with clear corneal media (Figure 1a). The infected cornea group (Figure 1b) showed mild opacity over the central cornea with mild stromal infiltrate. The treated cornea group (Figure 1c) showed persistent media opacity, caused by the drug-containing gel coating, at the corneal surface, but no obvious corneal stromal infiltrate was observed during the 24-h observation period.

3.2. Quantification of viable S. aureus bacteria from ex vivo cornea model

The anti-bacterial ability of the drug-loaded hydrogel was evaluated by quantifying the numbers of viable bacteria in samples of excised corneas (Figure 2). The results demonstrated that the levofloxacin-loaded hydrogel significantly inhibited the growth of *S. aureus*. The infected group (wound with *S. aureus*, abbreviated as WSA in Figure 2) showed an 8-fold increase in viable bacteria compared to controls. The infected with treatment group (wound with *S. aureus* and hydrogel treatment, abbreviated as WSAG in Figure 2) showed significantly less bacterial growth than the infected group (p < 0.05).

3.3. Anti-inflammatory effect of drug-loaded hydrogels

The anti-inflammatory effects of levofloxacin-loaded hydrogels were evaluated by measuring inflammation-related gene expression (i.e., mRNA expression of: *TNF-* α , *IL-1* α , *IL-6*, *IL-8*, *MMP-3*, and *MMP-9*; Figure 3). We found that the hydrogel treatment significantly lowered the expression of *TNF-* α , *IL-1* α , *IL-6*, and *IL-8* compared to the infected group. *MMP-3* expression was slightly, but not significantly, lower in the treated

с



b



Figure 1. External photographic images show time course of excised rabbit corneas, during a 24-h incubation period. (a) Control healthy corneas; (b) wounded corneas infected with *S. aureus*; (c) wounded corneas infected with *S. aureus* and treated with levofloxacin; each panel shows 2 representative corneas.







Figure 3. Inflammatory marker expression was reduced with levofloxacin-loaded hydrogel treatment. Relative mRNA expression levels of six inflammatory cytokines are shown for control group, infected group (WSA), and infected with treatment (WSAG) group. *p < 0.05, n = 3 in each group.

group than in the control and infected groups. *MMP-9* expression was similar in the infected and treated groups.

positive *S. aureus* (Figure 4b). The Gram stain of the infected group and infected with treatment group was presented (supplement 1).

3.4. Histological analysis

H&E staining showed no bacteria in the control or the treated group (Figure 4 a,c). In contrast, the infection group clearly showed gram-

3.5. Levofloxacin drug delivery in aqueous humor

We performed LC-MS/MS to analyze the aqueous humor of rabbit eyes at 1, 3, 6, 24, and 48 h after applying the thermosensitive

levofloxacin-loaded hydrogel (Figure 5). The peak observed at 3.1 min indicated that levofloxacin-loaded hydrogels released the drug into the aqueous humor at therapeutic concentrations.

4. Discussion

In this study, levofloxacin (10 mg/mL)-loaded thermosensitive chitosan-based hydrogel was prepared, as described in previous studies [31, 45]. Ameeduzzafar et al [48] used chitosan nanoparticles to encapsulate the antibacterial agent, levofloxacin, for treating ocular infections. Chitosan is widely used for sustained-released drug delivery, due to its high biocompatibility and non-immunogenic properties. Chitosan-containing hydrogel has several advantages: its mucoadhesive character increases the residence time; it is produced in a wide variety of sizes, from nanoparticles to centimeter-sized particles; and it is structurally similar to the extracellular membrane in humans. Chitosan-containing hydrogels showed high mechanical strength with an optimal gelation time, which might be particularly suitable for clinical applications.

Many studies have used *in vivo* animal models to investigate disease pathophysiology and the treatment effects of novel drugs [49, 50, 51]. However, *in vivo* studies require a large number of animals. Promoting the welfare of experimental animals by reducing the use of animals in research has become an important ethical issue, particularly when tolerance to the experimental treatment is unknown. Recently, some groups have turned their interest to the use of *ex vivo* models. Many *ex vivo* models retain the architecture and cellular stromal components observed *in vivo*. These models have been used to investigate wound healing processes and molecular microbial pathogenicity [52, 53, 54]. For these reasons, in the

present study, we implemented an *ex vivo* rabbit corneal keratitis model to evaluate the feasibility of the levofloxacin-loaded hydrogel. Pinnock et al [47] demonstrated that the scalpel-wounding method was suitable for inducing corneal infections in an *ex vivo* rabbit corneal model. We modified their method by using a fixation ring to mark the area for the scalpel wound and to restrict the area of infection and treatment to promote effective management of the interventions.

In our previous study [45], the levofloxacin-loaded hydrogel demonstrated a sustained-release profile, long-term antibacterial activity, and safety on cultured corneal epithelial cells. In the present study, the infected corneas were observed for 24 h after infection and treatment (Figure 2). The results revealed that the levofloxacin-loaded hydrogel significantly inhibited S. aureus growth in an infected wound. We also tested the anti-inflammatory effect of the levofloxacin-loaded hydrogel by evaluating the expression of inflammation-related mRNAs. We found that *TNF-* α , *IL-*1 α , and *IL-*8 expression levels were significantly lower in the treated group than in the infected and control groups. Moreover, IL-6 and MMP-3 were both expressed at lower levels in the treatment group than in the infection group, but the difference was not significant. TNF- α contributes to oxidative stress in sites of inflammation, which is one of the cytokines elevated under inflammatory processes. The IL family are known to be activated during immune system activation. Then MMP family are multifunctional enzymes that regulate both active and chronic inflammation. Assessing these inflammatory markers represented the underlying inflammatory process were ongoing. However, MMP-9 expression showed no differences between the infected and treated groups. Further examinations will be performed in order to verify the finding. Despite this, these results revealed that both the anti-bacterial



Figure 4. Histological analyses showed no visible bacteria after levofloxacin hydrogel treatment. Images of representative H&E stained sections of corneas in (a) the control group (20X and 40X), (b) the infected group (20X and 40X), and (c) the infection with hydrogel treatment group (20X and 40X). Red arrows: gram-positive *S. aureus*.



Figure 5. Representative chromatograms show baseline and 24-h levels of levofloxacin in aqueous humor. (a, *top*) diluted blank aqueous humor, spiked with 10 ng/ml levofloxacin; (*bottom*) diluted rabbit aqueous humor sample, containing 7.4 ng/ml levofloxacin. Levofloxacin peaked at 3.1 min; (b) levofloxacin concentrations in three samples at baseline and at 24 h after topical administration.

and anti-inflammatory therapeutic effects of levofloxacin could be achieved by loading the drug into a chitosan-gelatin-based hydrogel.

The three groups of *ex-vivo* corneal models were analyzed histologically to detect the presence of *S. aureus* with H&E and Gram stains, which are common methods for identifying bacteria in infected tissues. The histological results revealed a positive Gram stain for *S. aureus* in the infected group, but no bacteria were detected in the control or treated group. This result confirmed that the levofloxacin-loaded hydrogel effectively inhibited bacterial growth.

To test the ability of the hydrogel to release drug into the aqueous humor, we diluted the levofloxacin in the hydrogel to 10 ng/ml. The results showed that levofloxacin concentrations up to 7.4 ng/mL were present in a sample after 24 h after only one drop of instillation, which almost achieved the diluted concentration of 10 ng/mL loaded in the hydrogel. We selected this drug concentration, based on our previous study, which showed that this drug concentration had a good safety profile [45]. Many previous studies had examined the levels of drugs in the aqueous humor after the topical application of various levofloxacin concentrations (from 0.5% to 1.5%) in human eyes [55, 56, 57]. Our study was different from those studies in the time points measured and the frequency of drug instillations. In our study, we instilled a single dose and examined the aqueous drug concentration after 24 h. Most previous studies instilled more than one drop of levofloxacin and examined the aqueous drug concentration 1-6 h later. Our results showed that the levofloxacin-loaded hydrogel achieved the desired drug level, due to sustained release, even at 24 h after of a single dose.

5. Conclusion

We employed an *ex-vivo S. aureus* keratitis model to investigate the antibacterial activity of a levofloxacin-loaded hydrogel. We found that the hydrogel significantly inhibited bacterial growth in culture; indeed, we found no histological evidence of *S. aureus*. Moreover, the hydrogel had a significant anti-inflammatory effect. After applying one drop of the drug-loaded hydrogel, the drug was released into the aqueous humor and the concentration was maintained for at least 24 h. This study demonstrated that the hydrogel had sustained-released properties, which obviated the risk of contamination associated with frequent applications and diminished the problems of ocular surface evaporation. Our results with the *ex-vivo* model

suggested that the levofloxacin-loaded hydrogel could be useful for treating endophthalmitis or keratitis that develops after ophthalmic surgery.

Declarations

Author contribution statement

Yu-Fan Chang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yung-Hsin Cheng: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yu-Chieh Ko: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Shih-Hwa Chiou: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Catherine Jui-Ling Liu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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Y.-F. Chang et al.

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