Title: Safety profile of stromal hydration of clear corneal incisions with cefuroxime in the mouse model

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No proprietary or financial interest to declare from any of the authors.

Running title: Stromal hydration with cefuroxime

Key words: Cefuroxime, stromal hydration, clear corneal incision, endophthalmitis

Abstract

Purpose: The use of sutureless clear corneal incisions (CCI) for phacoemulsification is an established surgical technique, but the dynamic morphology of the wound and poor construction can lead to an increased risk of post-operative endophthalmitis. Stromal hydration with balanced salt solution (BSS) can improve the self-sealing status. Intracameral cefuroxime has reduced endophthalmitis rates. This study investigates the safety profile of stromal hydration with cefuroxime, as sequestering antibiotic at the wound may potentially provide added protection against infection.

Methods: MF-1 mice underwent bilateral CCI, followed by stromal hydration with either 5 µl of 10 mg/ml cefuroxime, cefuroxime-texas red conjugate (for detection using confocal microscopy), or BSS. Corneas were harvested from 1 hour to 12 weeks post-operatively; gross morphology, histology and apoptotic cell-death levels were investigated to determine the safety profile. Bactericidal activity of cefuroxime was assayed using homogenised whole cornea following stromal hydration at 1 hr, 24 hrs and day 7 against gram-negative *Escherichia coli*.

Results: Cefuroxime stromal hydration did not alter corneal morphology, with no

evidence of corneal scarring or vascularisation. Corneal histology and levels of apoptosis were minimal and comparable to the BSS groups up to 12 weeks. Confocal microscopy detected cefuroxime-texas red up to 1 week surrounding the corneal wound. Whole corneal tissue homogenates displayed bactericidal activity up to 24 hrs post operatively.

Conclusions: Stromal hydration of CCI with cefuroxime is safe in mouse corneas. A reservoir of antibiotic at the wound can potentially act as a barrier of defence against infection following cataract and associated ocular surgery.

Introduction

Cataract surgery is the most common operative procedure in the elderly population.[1] Endophthalmitis is a potentially blinding complication of cataract surgery caused by severe intraocular inflammation. It usually presents within a few days following cataract surgery, with 80% of cases within six weeks. The majority of culture-proven cases of post-operative endophthalmitis are due to gram-positive bacteria, namely *Staphylococcus epidermidis* and other coagulase-negative staphylococci, which reside on the ocular surface and are presumed to enter the eye through the surgical incision.[2] The use of clear corneal incisions (CCI) for cataract surgery has soared in popularity over the past 20 years. In the 2003 survey of practice styles and preferences of the American Society of Cataract & Refractive Surgeons (ASCRS) members, CCI were used by 72% of respondents as their preferred cataract surgery technique.[3] It has been suggested that there is an increased risk of post-operative endophthalmitis associated with the use of sutureless CCI.[4-6] This is due to the dynamic morphology of the wound, which may remain apposed in a well-pressurized eye, however natural fluctuation in intraocular pressure (IOP) within the physiological range of 5-40 mmHg can result in shifting and gaping of the wound edge.[7] This can lead to the ingression of ocular surface fluid including bacteria into the anterior chamber, with one retrospective cohort study showing that wound leak on day-1 post-op increased the risk of post-operative endophthalmitis by 44-fold.[8] The paracentesis wound has also been shown to leak using India ink, even in the absence of fluorescein leakage,[8] hence, attention to the construction of all

CCIs is imperative. To improve the self-sealing status of corneal incisions, intrastromal hydration of the wound can be performed with balanced salt solution (BSS).[9]

In 2007, the European Society of Cataract & Refractive Surgeons (ESCRS) Endophthalmitis Study Group reported that the use of intracameral cefuroxime at the end of surgery significantly reduced the occurrence of postoperative endophthalmitis.[10] The Cochrane review on perioperative antibiotics for prevention of acute endophthalmitis after cataract surgery found the results of the ESCRS study, the only prospective and randomised multicentre clinical trial, to provide the best evidence for antibiotic prevention of endophthalmitis.[1] In a recent survey investigating the adoption of intracameral antibiotic prophylaxis by 193 ophthalmic surgeons affiliated with private and public hospitals and clinics across Europe, 74% reported they always or usually used intracameral antibiotics (of which 82% used cefuroxime) in their cataract procedures.[11]

Cefuroxime is a bactericidal β-lactam second-generation cephalosporin. It acts by binding to specific penicillin-binding proteins (PBPs), which subsequently interferes with the peptidoglycan synthesis of the bacterial cell wall.[12] Cell lysis ensues following activation of bacterial cell wall autolytic enzymes such as autolysins. Cefuroxime is effective against aerobic gram-positive microorganisms (*Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes)*; aerobic gram-negative microorganisms (*Escherichia coli*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis, Haemophilus influenzae* and *Neisseria gonorrhoeae* including beta-lactamase-

producing strains); and spirochetes such as *Borrelia burgdorferi.*

Safe perioperative measures that can be implemented as prophylaxis against intraocular microbial contamination may further reduce the rates of post-operative endophthalmitis. In this study, we suggest stromal hydration of clear corneal incisions using cefuroxime may provide a safe and effective method of sequestering therapeutic levels of antibiotic at the wound site, with the potential of providing another barrier of defence against infection.

Materials and methods

Animals

Six-week-old MF-1 albino mice (from Harlan UK) were used in this study. They were maintained in a 12/12-hour light–dark cycle with free access to water and food at the Imperial College London animal facility according to standard protocols and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The local institutional review board approved this study.

Before performing surgical clear corneal incisions and stromal hydration, the mice were anaesthetised by intramuscular injection (0.01 ml/10 g body weight) of a mixture of 100 mg/ml ketamine hydrochloride (Narketan-10, Vétoquinol UK Ltd), 1 mg/ml medetomidine hydrochloride (Domitor®, Orion Pharma) and double distilled water at a ratio of 3:5:42, respectively. The anaesthesia was later

reversed by the intramuscular injection (0.01 ml/10 g of body weight) of 0.1 mg/ml atipamezole hydrochloride (Antisedan®, Orion Pharma). At the end of the follow-up period (1 hr, 24 hr, and weeks 1, 6 and 12 post-operatively), the mice were euthanized by intra-peritoneal injection of an overdose (80-90 mg/kg body weight) of sodium pentobarbital (Euthatal, Merial Animal Health Ltd). The eyes were then enucleated and the corneas excised. Non-operated corneas were used as the negative control.

Clear corneal incision and stromal hydration

The cornea was lubricated with a drop of Hypromellose 0.3% w/v (Martindale Pharma®, chemical name hydroxypropyl methylcellulose $C_{56}H_{108}O_{30}$) and excess fluid removed using a spear-shaped sponge (Harvard Apparatus). A 0.6 mm clear corneal incision was then placed in the temporal aspect of each cornea using an incisional micrometer diamond knife (DGH Technology Ltd). Intracorneal stromal hydration through the lips of the wound was performed using a 34-gauge Hamilton needle mounted onto polyethylene tubing (Intramedic^{TM}) and a gas tight 50 µl Hamilton glass barrel. The right corneal stroma was hydrated with 5 µl of 1 mg/0.1 ml (or 10 mg/ml) cefuroxime solution. The left eye was hydrated with 5 µl balanced salt solution (BSS©, Alcon Laboratories) to serve as a control.

Cefuroxime and Cefuroxime-Texas Red®-X conjugate

Cefuroxime sodium powder (GlaxoSmithKline) was dissolved in balanced salt

solution (BSS©, Alcon Laboratories) to a concentration of 1 mg/0.1 ml (or final concentration 10 mg/ml). This was made up fresh for each application. To assess sequestration of cefuroxime at the site of the CCI following stromal hydration, confocal microscopy was used to monitor the presence of cefuroxime-Texas Red®-X conjugate. To prepare the conjugate, 0.3 ml of 2 mg/ml stock solution of Texas Red®-X succinimidyl esters (Invitrogen Molecular Probes) dissolved in DMSO was agitated with 2.2 ml of cefuroxime (50 mg/ml stock) overnight at 4°C in the dark. This preparation was diluted with double distilled water to a concentration of 10 mg/ml cefuroxime-Texas Red®-X and 5 µl was used to stromally hydrate the cornea; and 5 µl of 10 mg/ml Texas Red®-X alone was also injected into the corneal stroma as a control. Stacked images of the cornea were taken using the Leica SP5 inverted confocal microscope at 1 hr, 24 hr and day 7. Total fluorescence intensity was measured (using Leica LAS AF Lite software) from each dissected cornea, n=3 at each aforementioned time point, providing a mean ± SEM fluorescence intensity per cornea for each treatment group.

Bactericidal assay

To determine the bactericidal activity of cefuroxime, harvested corneas at 1 hr, 24 hr and day 7 post-stromal hydration were homogenised by sonication (3 x 15 sec, XL-2000 Qsonica LLC) and the whole tissue homogenate was added to 50 µl gram-negative bacteria *Escherichia coli* DH5α cells incubated with 5 ml Luria-Bertani (LB) medium overnight at 37°C (n=3 corneas per time point). BSS-hydrated corneas at corresponding time points were used as the negative

control, alongside untreated corneas (n=3). Direct inoculation of 5 µl of 10 mg/ml cefuroxime was used as a positive control. Following this 10 µl of each culture was plated on agar plates overnight at 37°C to assess levels of bactericidal activity. The number of colony forming units (CFU) per ml was calculated by counting each colony on 3 agar plates per condition, using a Molecular Imager (Gel-Doc XR; Bio-Rad Laboratories, Munich, Germany) and determining the mean colony count per ml plated, divided by the total dilution factor.

Corneal histology and morphological studies

Freshly excised corneas were fixed in 4% paraformaldehyde (PFA) for 4-6 hours at room temperature, then washed three times in PBS, dehydrated through a graded ethanol series (50, 70, 90% and three times in 100%), and finally embedded in paraffin wax. Microtome sections of 5 µm were cut, mounted on Superfrost Plus slides and stained with H&E.

Corneal morphology and histology at various time points between 5 min and 12 weeks were taken using a Nikon 5100 mounted on a Stemi 2000 C 7:1 zoom stereomicroscope. The central corneal thickness was measured from the histological sections using ImageJ, and mean CCT ± SEM (n=3 at each time point per treatment group) was calculated.

TUNEL assay

Excised corneas with cefuroxime and BSS stromal hydration were fixed in 4%

PFA and embedded in wax as described above at 1 hr, and weeks 1, 6 and 12 post-operatively (n=3 at each time point per treatment group). Corneal sections were dewaxed by washing twice in histoclear (National Diagnostics), followed by two washes in 100% ethanol and once in 70% ethanol, before rinsing in deionized H_2O. Sections were digested with proteinase K (10 mg/ml) for 15 min. Following the manufacturer's instructions, the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore) was used to detect levels of apoptotic cell death to determine the safety profile of the stromal hydration.

Statistical Analysis

All results are expressed as mean ± SEM, unless specified. As sample sizes are small, non-parametric tests were used. Differences between control non-operated, BSS and cefuroxime-hydrated groups were assessed by pair-wise comparisons using the Mann-Whitney test or the Kruskal–Wallis analysis of variance if more than 2 groups were investigated, followed by Dunn's multiple comparison test where appropriate, unless otherwise stated. A p-value of <0.01 was considered statistically significant.

Results:

Stromal hydration of CCI with a 10 mg/ml concentration of cefuroxime did not adversely affect the cornea using an *in vivo* mouse model. The right and left eyes were stromally hydrated with 5 µl of 10 mg/ml cefuroxime and 5 µl of BSS, respectively. This volume of solution was chosen for intrastromal hydration as it

maximally hydrated the wound edges. Corneal morphology was comparable in both cefuroxime and BSS hydration groups at each time point from 1 hr to 6 weeks (Fig 1A). By week 6 there was no gross morphological difference in appearance between operated and non-operated eyes. There was no evidence of corneal scarring or vascularisation up to 12 weeks post-operatively.

Levels of apoptosis were assayed using TUNEL staining in both BSS and cefuroxime stromal hydration groups up to 12 weeks post-operatively. From day 1 through to week 12 there was minimal evidence of cell death, and each cornea was comparable in both groups to a non-operated eye (Fig 1C).

Corneal histology showed an increase in corneal thickness at 1 hr post-stromal hydration in both the BSS (mean 121.3 ± 2.6 μm) and cefuroxime (mean 121.0 ± 1.6 μm) groups. Each group had comparable central corneal thickness (CCT) at each time point investigated (Fig 2), with no statistical difference ($p>0.95$): at one week post-operatively the mean CCT was 98.0 ± 1.4 μm in the BSS and 96.3 ± 1.2 μm in the cefuroxime group; at 6 weeks post-operatively the mean CCT was 93.7 ± 2.6 μm in the BSS and 92.0 ± 2.1 μm in the cefuroxime group; and at 12 weeks the mean CCT was 87.3 ± 2.1 μm in the BSS and 88.3 ± 1.7 μm in the cefuroxime group similar in thickness to an untreated cornea, mean CCT 87.7 ± 1.69 μm (no statistical difference, $p>0.98$).

Cefuroxime-texas red conjugate and texas red alone was detected by confocal microscopy up to 1 week surrounding the corneal wound (Fig 3). Cumulative levels of fluorescence were measured from stacked images of each dissected

cornea stromally hydrated with cefuroxime-texas red (mean 42.5 x 10^5 ± 4.4 x 10^4 fluorescence intensity/cornea) and texas red alone (mean 35.1 x 10^5 ± 4.0 x 10^4 fluorescence intensity/cornea) at 1 hour post-operatively. This dropped by 42.1% by day 1 post-operative in the cefuroxime-texas red group (mean 17.9 x 10^5 ± 5.2 x 10^4 fluorescence intensity/cornea) and 42.7% in the texas red group (mean 15.0 x 10^5 ± 8.0 x 10^3 fluorescence intensity/cornea). At one week post-operatively, in the cefuroxime-texas red group (mean 4.1 x 10^5 ± 1.6 x 10^4 fluorescence intensity/cornea) less than 10% fluorescence intensity was detected compared to the 1 hr post-operative time point. This was similar in the texas red group (mean 3.6 x 10^5 ± 8.0 x 10^3 fluorescence intensity/cornea). No fluorescence was seen in the BSS hydrated corneas, which were used as the negative control.

Microbiological tests to assess the bactericidal activity of cefuroxime in the stromally hydrated cornea showed a significant efficacy at 1 hr post procedure (2.1 x 10^6 ± 4.8 x 10^6 CFU/ml) compared to the BSS hydrated corneas at the same time point (7.6 x 10^8 ± 3.2 x 10^7 CFU/ml) and non-operated cornea controls (7.7 x 10^8 ± 1.3 x 10^7 CFU/ml), $p<0.01$ (Fig 4). Although less than at 1 hr, a significant degree of bactericidal activity was still seen at 24 hrs (2.4 x 10^8 ± 2.7 x 10^7 CFU/ml) compared to the BSS hydrated corneas (7.5 x 10^8 ± 4.1 x 10^7 CFU/ml) and negative control group, $p<0.01$. By day 7 there was no difference in cefuroxime hydrated corneas (7.4 x 10^8 ± 3.9 x 10^7 CFU/ml) compared to the BSS hydrated corneas (7.2 x 10^8 ± 6.6 x 10^7 CFU/ml) and non-operated cornea controls, $p<0.95$. When 5 µl of 10 mg/ml cefuroxime was added directly to the *Escherichia coli* DH5α cells, no growth was seen in the agar plates, suggesting

that this dose is bactericidal and optimum for administration into the eye (Fig 4).

Discussion:

Stromal hydration of CCI with 10 mg/ml cefuroxime appears safe in mouse corneas with demonstrable levels up to seven days post-operatively. This is consistent with a recent study which found that stromal hydration is detectable for at least one week in patients undergoing cataract surgery performed through CCI confirmed by 3-D corneal and anterior segment OCT. [13] Fine et al. used a Zeiss Visante anterior segment OCT to detect stromal swelling at 24 hours post-cataract surgery but did not examine any later timepoints.[14] A reservoir of antibiotic at the wound could act as a barrier of defence against infection, with the potential to reduce the risk of post-operative endophthalmitis following cataract and associated ocular surgery.

Pharmacokinetic studies have shown that cefuroxime is excreted unchanged in the urine and has a half-life of 1-2 hours.[15] A non-randomized observer-masked trial was conducted to investigate the safety and kinetics of prophylactic intracameral cefuroxime in patients who underwent uneventful phacoemulsification.[16] Patients received a dose of 1 mg cefuroxime in 0.1 ml saline 0.9% into the anterior chamber at the end of surgery. One hour later, following aqueous sampling, a four-fold reduction in the levels of cefuroxime was detected from 2.74 mg/ml to 0.75 mg/ml. No further time points were measured and as there were no reports of aqueous turnover following cataract surgery, the true elimination rate could not be fully ascertained. From our study, the detection

of cefuroxime-texas red conjugate and corresponding bactericidal activity up to 24 hrs, suggests adequate concentrations of cefuroxime were present in the cornea following surgery. Bacterial colonies were detected at both time points, compared to the direct administration of cefuroxime to the gram-negative bacteria *Escherichia coli,* but this is likely due to the dilution effect caused by using the whole cornea tissue homogenate. Nonetheless, presence of low levels of antibiotic had some inhibitory action on the growth of bacteria, and in practice, if concentrated around the wound edges will have a more potent protective effect. The main limitation of this study is the lack of serial drug level monitoring in the stroma, anterior chamber and tears. Further studies are warranted to measure the release of cefuroxime from the stroma to the aqueous humour and into the tears over time in order to determine the true elimination rate from the cornea, and whether its minimum inhibitory concentration (MIC) levels could be achieved for *Staphylococcus epidermidis* and other relevant bacteria associated with endophthalmitis.

A recent study investigated the penetration of bevacizumab administered to BALB/c mouse eyes topically with a denuded corneal epithelium or via subconjunctival injection with an intact cornea.[17] At 24 hours post administration there was evidence of widespread intracorneal diffusion with even stromal distribution throughout the whole cornea using both methods. Bevacizumab remained present in the central cornea for up to 14 days, declining at 21 days from the periphery. Due to its large molecular weight, 149 kDa, it was hypothesized that bevacizumab was removed by diffusion from the corneal periphery in a centripetal pattern slowly over weeks. In contrast, cefuroxime is a

small molecule, formula $C_{16}H_{16}N_4O_8S$, with a molecular mass of 424.386 Da, but this compound may act in a similar manner reducing in levels through diffusion secondary to its high concentration gradient in the stroma over time. Stromal hydration with cefuroxime would allow a known concentration of drug to be delivered to the cornea, which could act as a slow release mechanism at the wound site and into the anterior chamber. In theory, stromal hydration may be useful for patients where compliance may be an issue, as a bolus may cover the patient over the first few days up to one week post-operatively.

There has been a reluctance to adopt the use of intracameral antibiotics, namely cefuroxime (0.1 mL of 10.0 mg/mL solution), over the past few years despite its result in a 5-fold reduction in the incidence of postoperative endophthalmitis. Fifty-five percent of surgeons surveyed in the United Kingdom in 2008 reported using intracameral cefuroxime, as compared with 10% in 2005.[18] The unwillingness was mainly due to the concern of unintended dilution errors, endothelial toxicity and possible bacterial contamination. Previously, there were no ready-to-use ocular formulations of cefuroxime available, hence the 10 mg/ml solution was prepared in hospital pharmacies and stored at 4°C for a limited time or reconstituted by the surgeon in the operating room, which risked mistakes during the dilution. A series of 6 cases was published by Delyfer *et al.* 2011 documenting the inadvertent administration of high dose (100 mg/ml) intracameral cefuroxime at the end of uneventful cataract surgery, secondary to misunderstanding of the dilution protocol.[19] Each case resulted in moderate anterior inflammation and fibrin formation consistent with a mild form of toxic anterior segment syndrome or toxic endophthalmitis, which had resolved by day

5. All patients developed extensive macular oedema with an associated serous retinal detachment on day one post-operatively, which significantly improved in the first week. At six weeks CCT, endothelial cell density and macular anatomy had returned to normal in all patients consistent with those observed after uneventful phacoemulsification. Two-thirds of the surgeons not using intracameral cefuroxime stated they would adopt this step if a commercial preparation were available.[18] A one-step reconstitution of cefuroxime (Aprokam®) is now available and this may encourage the use of intracameral cefuroxime and its further application in stromal hydration of the cornea.[20] However, a pre-filled single-use syringe containing 10 mg/ml cefuroxime would be ideal to eliminate any preparation and dosage errors.

Our experiments show that there were minimal levels of cell death in the cefuroxime and BSS stromal hydration groups, both comparable to non-operated eyes. There have been many studies assessing the corneal endothelial changes after intracameral cefuroxime following cataract surgery. In a recent study endothelial cell density (ECD), coefficient of variation (CoV), and hexagonality were evaluated at baseline and 1 week, 1 month, and 3 months following intracameral cefuroxime (1 mg/0.1 ml) or vancomycin (1 mg/0.1 ml) after cataract surgery.[21] The ECD was significantly reduced at 1 week post-surgery, after which it stabilized but overall it decreased by a mean of 12.1% at 3 months. There was no significant difference in the CoV or hexagonality preoperatively and at 3 months postoperatively.

There has been little advance in perioperative techniques for the prevention of

acute endophthalmitis over recent years. Prior to the 2007 ESCRS Endophthalmitis Study, which demonstrated the efficacy of using intracameral antibiotics for reducing endophthalmitis, only preoperative disinfection by povidone–iodine could reach the level II rating of scientific evidence.[1, 22] Other reported prophylactic interventions, such as antibiotic-containing irrigating solutions, the use of intraoperative heparin and postoperative subconjunctival antibiotic injection received the lowest clinical recommendation based on weak evidence justifying their use.[22] This preclinical study shows proof-of-concept for developing a new perioperative prophylactic intervention, which uses the same concentration of intracameral cefuroxime for stromal hydration of the surgical wounds. Anectodotally, this technique is in use by some cataract surgeons, however, the method has not been previously published in the literature. There is contention about the transferability of mouse corneal data to humans, but this study does confirm the safety of cefuroxime intrastromal hydration in murine models. Clinical trials would be required to assess the safety in patients with a view to evaluating this procedure using large patient groups to compare rates of post-operative endophthalmitis following cataract surgery.

Acknowledgements

We would like to acknowledge the National Institute for Health Research UK (Moorfields Eye Hospital and UCL Institute of Ophthalmology Biomedical Research Centre) and the Eli Webster Ophthalmic Charity Fund, Charing Cross Hospital, London, UK.

No competing financial interests exist for any author.

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Legends

Figure 1. Morphology, histology and cell death assay comparing stromal hydration of a clear corneal incision with cefuroxime (CEF 10 mg/ml) or BSS in the mouse eye post-operatively. (A) Morphology of a non-operated mouse eye followed by images of a CEF hydrated cornea at 1 hr, day 1 and week 6 post-operatively. Normal morphology can be seen at week 6, comparable to the BSS hydrated cornea shown at the end of the panel. No evidence of any corneal vascularisation or scarring. (B) Histology of the central cornea of an non-operated mouse eye followed by comparable sections of CEF and BSS hydration groups at day 1 (maximum stromal hydration seen), and week 1, 6 and 12 post-operatively. (C) Cell death assay on an non-operated mouse central corneal section was comparable to CEF and BSS hydration groups at all time points up to 12 weeks post-operatively with minimal to no cell death noted.

Figure 2. Graphical representation showing the mean ± SD reduction in central corneal thickness (CCT in μm) following stromal hydration with cefuroxime (CEF, 10 mg/ml) or BSS over time post-operatively from 1 hr up to 12 weeks (n = 3 per time point per group; WT, control non-operated eye).

Figure 3. Fluorescence levels of cefuroxime-texas red conjugate following stromal hydration of the corneal wound. Stacked confocal fluorescent merged with bright field images of the cornea hydrated with cefuroxime-texas red conjugate (CEF-TR), texas red alone (TR) and BSS at 1 hr, 24 hrs and day 7 post-operatively. Corresponding graph showing the mean fluorescence intensity x 10^5 per whole cornea ± SEM (n = 3 per time point per group) of CEF-TR and

TR.

Figure 4. Graph to show the levels of bacteria (colony forming units [CFU]/ml) following inoculation with cefuroxime-stromally hydrated corneas (CEF) compared to BSS-hydrated corneas (BSS) at 1 hr, 24 hrs and day 7 post-operatively. Negative control (-) was non-operated cornea and the positive control (+) was 5 µl of 10mg/ml cefuroxime.