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# Bacterial corneal ulcers. Intrastromal treatment with fourth-generation quinolones.

--Manuscript Draft--

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| **Abstract:** | ABSTRACT<br>Aims  Determine the therapeutic efficacy of intrastromal vs. topical fourth-generation quinolones in the treatment of corneal ulcers due to Pseudomonas aeruginosa (P. aeriginosa).<br>Methods  We develop an experimental, comparative in vivo study using rabbits (N=20) to determine the effect of intrastromal injection of quinolones vs. topic treatment. Mechanical debridement of the cornea was performed to twenty New Zealand white rabbits. One drop of P. aeruginosa culture was instilled. The rabbits were randomized to 5 groups. Groups A and E received intrastromal treatment: 2 doses of moxifloxacin 0.5 µg/0.02 cc every 3 days. Groups B and D received topical moxifloxacin every 4 hours. Treatment began 48-72 hours after induction of infection. Macroscopic changes were documented by digital photography. Corneoscleral dissection, histopathological study and alpha-smooth muscle actin (α-SMA) immunofluorescence were performed. Z-scores to integrate macroscopic and microscopic findings and comparison between topic and Intraestromal was done using Mann-Whitney test. Significance was set at $p<0.05$.<br>Results  The digital photographs showed varying improvements in corneal ulcers. The statistical analysis showed a risk ratio of 0.88 between groups, suggesting a 22% improvement with intrastromal treatment; however, no statistical significance was observed.<br>Conclusion  Intrastromal treatment is associated with less corneal damage on |

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|---|---|
| | histopathological and macroscopic study. However, the infection/healing process by α-SMA was evident in groups that received topical and intrastromal treatment, suggesting that this marker may be used at later stages in order to determine differences in therapeutic response in animal models. |
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Title: Bacterial corneal ulcers. Intrastromal treatment with fourth-generation quinolones.

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**Key words**: Intrastromal treatment, quinolones, corneal ulcer, *P. aeruginosa,* immunofluorescence.

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**Short title**: Intrastromal treatment of corneal ulcers.

## ABSTRACT

**Aims** Determine the therapeutic efficacy of intrastromal vs. topical fourth-generation quinolones in the treatment of corneal ulcers due to *Pseudomonas aeruginosa (P. aeriginosa)*.

**Methods** We develop an experimental, comparative *in vivo* study using rabbits (N=20) to determine the effect of intrastromal injection of quinolones vs. topic treatment. Mechanical debridement of the cornea was performed to twenty New Zealand white rabbits. One drop of *P. aeruginosa* culture was instilled. The rabbits were randomized to 5 groups. Groups A and E received intrastromal treatment: 2 doses of moxifloxacin 0.5 µg/0.02 cc every 3 days. Groups B and D received topical moxifloxacin every 4 hours. Treatment began 48-72 hours after induction of infection. Macroscopic changes were documented by digital photography. Corneoscleral dissection, histopathological study and alpha-smooth muscle actin (α-SMA) immunofluorescence were performed. Z-scores to integrate macroscopic and microscopic findings and comparison between topic and Intraestromal was done using Mann-Whitney test. Significance was set at $p<0.05$.

**Results** The digital photographs showed varying improvements in corneal ulcers. The statistical analysis showed a risk ratio of 0.88 between groups, suggesting a 22% improvement with intrastromal treatment; however, no statistical significance was observed.

**Conclusion** Intrastromal treatment is associated with less corneal damage on histopathological and macroscopic study. However, the infection/healing process by α-SMA was evident in groups that received topical and intrastromal treatment, suggesting that this marker may be used at later stages in order to determine differences in therapeutic response in animal models.

## INTRODUCTION

The corneal epithelium, with its lipophilic nature and highly developed intercellular junctions, interdigitated membranes, microscopic protrusions (microvilli), tight junctions, desmosomes and gap junctions, is an extremely stable anatomical structure. The epithelial barrier protects against the penetration of drugs and water soluble electrolytes, limiting absorption, while the hydrophilic corneal stroma acts as a barrier for drugs delivered in lipid emulsions, sometimes preventing medicinal products from acting on the corneal stroma. Moreover, parenteral or subconjunctival administered drugs do not easily reach the stroma due to the absence of blood vessels.[1,2]

Topical administration is simple, but penetration of intraocular tissue and deep stroma is poor due to the brief contact with the eye surface and the lipophilic/hydrophilic corneal barrier.[2]

Intrastromal administration of cefuroxime (250 µl/ml) has been used to treat a case of crystalline keratopathy caused by *Streptococcus parasanguis* infection. In this case, the intrastromal approach proved beneficial in treating bacterial corneal infections.[3]

Fluoroquinolones are bactericidal agents that can penetrate the corneal stroma to act on 2 DNA gyrase enzymes (topoisomerase II and topoisomerase IV), causing the DNA to become over-wound, impeding replication, transcription, recombination and repair of the strand, and leading to bacterial death.[4]

Studies have reported that intrastromal second-generation fluoroquinolones were effective in healing corneal ulcers due their bactericidal action. Ciprofloxacin was shown to increase the number of keratocytes and mixed inflammatory cell infiltrate at a minimum inhibitory concentration (MIC) of < 1 µg: corneal healing was faster, but was still incomplete after 7 days.[5]

Corneal fibroblasts grown in presence of transforming growth factor beta (TGFβ) under serum-free conditions express α-smooth muscle actin, a biochemical marker for myofibroblasts which are implicated in corneal remodeling.[6]

Studies have reported that the expression of α-SMA (α-smooth muscle actin) is absent in corneal fibroblasts with successful reconstruction, and normal corneas. In contrast, the α-SMA protein is expressed in corneas with partial repair, so this indicates that a complete remodeling is associated with absence of myofibroblasts, and an incomplete remodeling or presence of secondary corneal fibrosis damage allows the persistence of myofibroblasts.[7]

The corneal infections pose a threat to the vision and whose clinical course varies depending on the bacterial virulence, condition of the cornea prior to infection, duration of the infection, and the immune status of the patient. Does not exist any comparative studies in the literature that demonstrates the effectiveness of intrastromal vs. conventional topical administration of antibiotics such as fourth-generation quinolones (moxifloxacin) in the treatment of bacterial ulcers. Therefore, we hypothesized, the intrastromal administration of 2 doses moxifloxacin at 0.5 µg/0.02 cc (4 µg/0.1 cc) every 3 days to treat corneal ulcers caused by *Pseudomonas aeruginosa* infection would improve or halt clinical progression of corneal damage, with fewer local side effects and cause less corneal remodeling compared with conventional topical treatment.

## MATERIALS AND METHODS

### Animals

This was an experimental, comparative *in vivo* study in 20 New Zealand white rabbits obtained from the vivarium of the Hospital Dr. Luis Sánchez Bulnes of the Association to Prevent Blindness in Mexico, Mexico City-DF. The animals were handled in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology. The rabbits weighed between 1,800 and 2,000 g, and were randomized to 5 groups: A, B, C, D or E, with 7 rabbits in groups A and B and 2 in each of the remaining groups. Identifiable human material or data

were used in this study, and permission to access this information was approved by the independent ethics committee.

## Identification of study groups

Eyes were randomized to receive either intrastromal or topical fourth-generation quinolones, depending on their assigned group. The antibiotic was loaded into sterile tuberculin syringes labeled with the letter of each group. Sterile eye drop bottles were loaded with the topical antibiotic, also labeled with the letter of each group. Groups A and E received 2 doses of intrastromal moxifloxacin 0.5 µg/0.02 cc (4 µg/0.1 cc) every 3 days (Figure 1A), while groups B and D received topical instillation of moxifloxacin every 4 hours. Treatment was started 48 - 72 hours after onset of infection. The animals were sacrificed with a rapid intracardiac administration of sodium thiopental (203 ml) 7 days after the start of antibiotic treatment.

## Standardization of the Inoculation process

*Culture of pathogenic strains*

Bacterial suspension of *Pseudomonas aeruginosa* obtained from human bacterial keratitis at a concentration of 75 X $10^6$ CFU per ml saline solution after 24 hours of culture in MH medium.

*Mechanical corneal debridement with scalpel*

After induction of general anesthesia with intramuscular administration of ketamine hydrochloride (40 mg/kg) (Rotexmedica-GMBH, Germany) and xylazine hydrochloride (7 mg/kg), topical anesthesia with proparacaine was administered and a 7.5 mm corneal trephine was centered over the limbus. Slight pressure was applied to the trephine to demarcate a circle on the corneal surface, and 70% isopropyl alcohol drops were instilled inside this area (Figure 1B). The corneal trephine was withdrawn and the surface of the cornea was rinsed with abundant 9% saline solution (Figure 1C). Immediately following this, mechanical debridement of the cornea was performed within

the marked areas using a # 11 Bard-Parker scalpel (Figure 1D). The right eye of each rabbit was designated as the study eye, and received either topical or intrastromal antibiotic treatment with moxifloxacin.

Inoculation of the pathogen After donning sterile surgical gloves and gowns, researchers collected the pathogens on the blade of the scalpel and placed them on the debrided area of the cornea of both eyes.

**Measurement of the corneal ulcer**

Photographs of each eye in each experimental group were taken using an Olympus® digital camera (model 3040-Z, Tokyo, Japan) at a resolution of 2048 x 1040 pixels. Photographs were taken every 3 days to determine the progress of the epithelial defect.

Photographs were processed using image analysis software (Scion Image®, Friederich, MD). Each photograph was scaled to the size of the area of debridement at time point 0 (7.5 mm).

**Anatomical pathology study**

An incision was made at 3 mm from the limbus in all eyes to remove the corneoscleral button. The buttons were fixed in 10% buffered formaldehyde for 24 hours, and were sent to the Pathology Department of the Hospital Dr. Luis Sánchez Bulnes, Association to Prevent Blindness in Mexico. Mexico City-DF, where they were dehydrated in increasing concentrations of alcohol, rinsed in xylol and mounted in paraffin blocks. They were subsequently cut into 3-micron sections using a microtome. The sections were then deparaffinized, placed on histology slides and stained with periodic acid–Schiff (PAS) and H&E and Gomori trichrome for histological study under a light microscope (Nikon. LABOPHOT-2. Japan).

**Alpha-smooth muscle actin immunofluorescence (α-SMA)**

The corneoscleral sections were fixed in 4% formaldehyde at room temperature. After serial washing, the sections were incubated overnight with mouse monoclonal α-smooth muscle actin antibody (1:200). Primary antibody bind-

ing was revealed using Cy3. Finally, the sections were mounted using DAPI/antifade solution for cellular nuclei staining. The sections were evaluated along the entire tissue sample using an Axioskop 2 mot plus confocal microscope (Carl Zeiss, USA) to obtain a qualitative measurement of α-SMA content (1 = present. 0 = absent). Photographs were taken of randomly selected areas using a digital camera (SpotCam RT KE Diagnostic Instruments Inc., Sterling Heights, Michigan, USA) attached to an ELYRA confocal fluorescence microscope (Carl Zeiss, USA). Cells stained with α-SMA were evaluated in 10 randomly selected areas and counted at 200X magnification to obtain a representative sample. Autofluorescent signal was determined using corneoscleral samples without primary antibody (negative controls). Ulcerated corneas from rabbits without treatment were used as positive controls of α-smooth muscle actin staining.

**Data collection and statistical analysis**

The measurements obtained were entered into a database for statistical analysis (Minitab® v.17.1.0 for Windows, Minitab Inc., USA). Quantitative variables were analyzed using the mean and standard variation of measurements from each group. The data obtained from the different treatment techniques were grouped using z-score statistics, assigning a weight of 12.5% to each cell culture, 50% to macroscopic findings, 40% to histopathological findings, and 10% to immunofluorescence findings. Analysis was performed using the Mann-Whitney non-parametric test. The relative risk was also determined, comparing intrastromal and topical treatment groups using the following formula: [number of positive cases treated intrastromally/(total cases treated intrastromally)]/[number of positive cases treated topically/(total cases treated topically)]. Anova and Bonferroni tests and a Cox model based on the slope value were used to analyze the rate of epithelial defect resolution in groups with corneal ulcers. Significance was set at $p<0.05$.

**RESULTS**

The clinical evaluation, based on the chronological series of digital photographs taken from day 0 (corneal abrasion) to day 7 of intrastromal and topical treatment, showed varying improvements in corneal ulcers, according to the type of treatment used (Figure 2).

Normalized evolution of the epithelial defect differed between study groups and controls. A comparison of study groups (intrastromal and topical) every 3 days using multiple comparison methods (F) showed statistically significant differences in epithelial defect remodeling. Table 1 shows the mean variations in the area of epithelial defect, which was significantly reduced in all groups ($F = 8.861$; $p = 0.001$). Notwithstanding, re-epithelialization was more advanced in rabbits treated with intrastromal moxifloxacin. Statistically significant differences were also observed between the treatments used in each group ($F = 4.643$, $p = 0.047$).

Histological study was performed of the sections. *Group A* (intrastromal treatment) showed a stratified cuboidal epithelium in the peripheral cornea. Below the epithelium, fibroblast proliferation accompanied by blood vessels and some inflammatory cells were observed, as well as intrastromal edema between the basal and the middle layer. The stroma was formed of layers of homogenized connective tissue with blood vessels containing red blood cells, proliferation of fibroblasts and scant mixed inflammatory cell infiltrate. In the periphery, congested blood vessels and inflammatory infiltrate can be seen adjacent to the limbus. *Group B* (topical treatment) showed a stratified cuboidal epithelium with mild edema in the basal layer. The stroma was formed of homogenized layers, with proliferation of fibroblast and scant presence of inflammatory cells (lymphocytes). *Control group* samples showed exclusively peripheral non-keratinized stratified squamous epithelium. In the central area, an ulcer with necrosis of superficial corneal lamellae and considerable mixed inflammatory cell infiltrate with eosinophils was observed. Extensive homogenization and necrosis was observed in the posterior stroma. Descemet's membrane was seen as a wavy formation, with inflammatory fibrinous exu-

date on the posterior surface and scant endothelial cells in the periphery (Figure 3).

A greater number of samples cultured from the groups treated with intrastromal moxifloxacin were negative at third day after inoculation. Nevertheless, better response was obtained with intrastromal treatment (22.22% reduction) compared with topical application. The group treated with intrastromal moxifloxacin showed more focal edema and fibroblasts compared with the topical moxifloxacin group, the latter showed greater presence of blood vessels.

Statistical analysis of the findings of the macroscopic diagnosis showed a relative risk (RR) of 0.88 in the intrastromal group compared with the topical treatment group, suggesting a 22% improvement with intrastromal treatment. The integrated analysis of findings showed no statistically significant differences (p=0.289).

Presence of α-smooth muscle actin was observed with both treatments; abundant α-smooth muscle actin immunofluorescence was observed in corneas from rabbits with untreated corneal ulcers (Figure 4A). All (100%) samples from the groups treated both intrastromally and topically were positive for α-smooth muscle actin. The intensity of the α-SMA signal was similar in both groups, under equal conditions (Figure 4B and 4C).

## DISCUSSION

Ophthalmic antibiotics are currently administered in a variety of ways, the most common being topical application, which allows the medication to penetrate as far as the aqueous humor to achieve adequate minimum inhibitory concentration (MIC) at the deepest site of inflammation. The extent of penetration of the antibiotic will depend on its electrostatic load, its ability to bind to the inflammatory proteins, its anti-bacterial activity, and the sensitivity of the infectious agent.[5]

Intracorneal microinjection of long-acting steroids has been used in the treatment of corneal graft rejection. Although a different drug was used in our series, namely an antibiotic, the intrastromal approach has been shown to be both effective and free from ocular side effects. This same strategy can be used to treat different ocular diseases; administration is easy and inexpensive, and low doses are therapeutically effective.[6]

*In vitro* studies have reported that fourth-generation fluoroquinolones can be more beneficial in the treatment of bacterial keratitis, although this needs to be confirmed in clinical trials [7,8]. In our study, we observed that these compounds (in the form of moxifloxacin) were effective in resolving ulcers, and that intrastromal administration of the antibiotic proved effective, with scant adverse effects, such as intrastromal edema between the basal and the middle layer, stromal blood vessels containing red blood cells, and scant mixed cell inflammatory infiltrate. Clinical improvement was observed in all cases.

Bactericidal antibiotics, such as fluoroquinolones, are effective at minimum inhibitory concentrations and have low toxicity.[8,9] For this reason, ciprofloxacin was the antibiotic of choice in our previous study of corneas inoculated *with P. aeruginos*a, even though it was not 100% effective.

Similar levels of α-SMA expression were observed in both topical and intrastromal groups. Smooth muscle actin is one of a group of cytoplasmic actins, which make up the microfilament system of the cytoskeleton and are highly conserved in mammals.[8] We determined the presence of α-SMA in the cytoskeleton, a marker of the myofibroblasts responsible for contraction of corneal ulcers, and determined its value as a marker of therapeutic response.[9] Absence of α-SMA is associated with absence of myofibroblasts,[7] and occurs at late stages of the healing process. Interestingly, we found abundant levels of α-SMA in the corneas of untreated rabbits, confirming its role as a marker of corneal inflammation. Marker levels coincided with the timing of histopathological, and even macroscopic, changes. This finding confirms our hypothesis that α-SMA is a marker of acute inflammation that

responds to early repair processes. However, after the start of both treatments (topical vs. intrastromal), we observed a marked decrease in α-SMA levels in both groups. This suggests that both treatments were effective in reducing the damage caused by both the injury and the infection. One of the limitations of our study lies in the fact that ELYRA light microscopy is a qualitative technique, and cannot be used to detect intergroup differences. We believe this marker (α-SMA) should be determined using quantitative techniques, such as real time RT-PCR, at later stages of the infection/healing process in order to determine differences in therapeutic response.

## CONCLUSION

The value of this study was to explore an alternative (intrastromal) route of administration in the treatment of corneal ulcers caused by Pseudomonas aeruginosa infection, to determine the MIC of 0.5 µg/0.02 cc moxifloxacin (4 µg/0.1 cc), and to maximize stromal penetration of the antibiotic. Likewise, our findings suggest that intrastromal treatment is associated with less corneal damage on histopathological and macroscopic study.

## WHAT WAS KNOWN

- Treatment of corneal ulcers is usually done by topic antibiotic treatment. However, topic administration has several disadvantages, including the poor penetration in the intraocular tissue and deep stroma due to the brief contact with the eye surface and the lipophilic/hydrophilic corneal barrier.
- Therapeutic strategies for corneal ulcers by *P. aeuriginosa* using intraestromal administration of fluoroquinolones, as well as its effect on corneal α-smooth muscle actin (α-SMA) —a recently described marker of corneal acute inflammation— has not been studied previously.

## WHAT THIS PAPER ADDS

- The intraestromal administration of 2 doses fluoroquinolones was effective in resolving ulcers caused by *Pseudomonas aeruginos*a, with scant adverse effects using MIC of 0.5 µg/0.02 cc moxifloxacin.
- Determination of α-SMA in corneal tissues may help to determine inflammatory status associated to corneal ulcers by *P. aeuriginosa* and to monitor the response to treatment.


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**COMPETING INTERESTS:** None.

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7 Espana EM, Ti S-E, Grueterich M, *et al.* Corneal stromal changes following reconstruction by ex vivo expanded limbal epithelial cells in rabbits with total limbal stem cell deficiency. *Br J Ophthalmol* 2003;**87**:1509–14.

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refractive surgery: the role of myofibroblasts. *Prog Retin Eye Res* 1999;**18**:311–56.

## LEGENDS

Figure 1 A. Slight pressure was applied to the trephine to demarcate a circle on the corneal surface, into which drops of 70% isopropyl alcohol were instilled.

Figure 1 B. The corneal surface was washed with abundant sterile 9% saline solution.

Figure 1 C. Mechanical debridement was performed within the marked areas using a # 11 Bard-Parker scalpel.

Figure 1 D. Groups A and E received intrastromal treatment with 0.5 µg/0.02 moxifloxacin (4 µg/0.1 cc).

Figure 2. Digital photographs taken from day 0 (corneal abrasion) to day 7 of intrastromal and topical treatment.

Figure 3. Histological sections, group A (intrastromal treatment), group B (topical treatment) and group C (controls).

Figure 4. A, B, C. Alpha-SMA immunofluorescence in the corneas of rabbits with untreated, intrastromal and topical treated corneal ulcer due to *Pseudomonas aeruginosa* infection.

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Table



Table 1. Therapeutic effect on the area of the epithelial defect, in increments of 2 mm, after mechanical corneal injury.

| | | Follow up (days) | | |
|---|---|---|---|---|
| | Pre-treatment | 3 | 6 | 7 |
| | Mean (SD) | Mean (SD) | Mean (SD) | Mean (SD) |
| Corneal ulcer without treatment | 30.3 (2.1) | 34.1 (3.0) | 39 (0.8) | 40.6 (1.4) |
| Intrastromal Moxifloxacin | 40.2 (1.4) | 35.2 (3.0) | 24.7 (6.5) | 21.1 (5.7) |
| Topic Moxifloxacin | 40.1 (1.8) | 36.7 (2.3) | 37.0 (1.7) | 34.7 (1.9) |
| Antibiotic F= 4,643 (p=0,047). Follow up: F= 8,861 (p=0,001). | | | | |
| Bonferroni test post-hoc: Topic vs Intrastromal: p= 0,2. | | | | |
| SD: Standard Desviation | | | | |

Figure

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Figure 1A

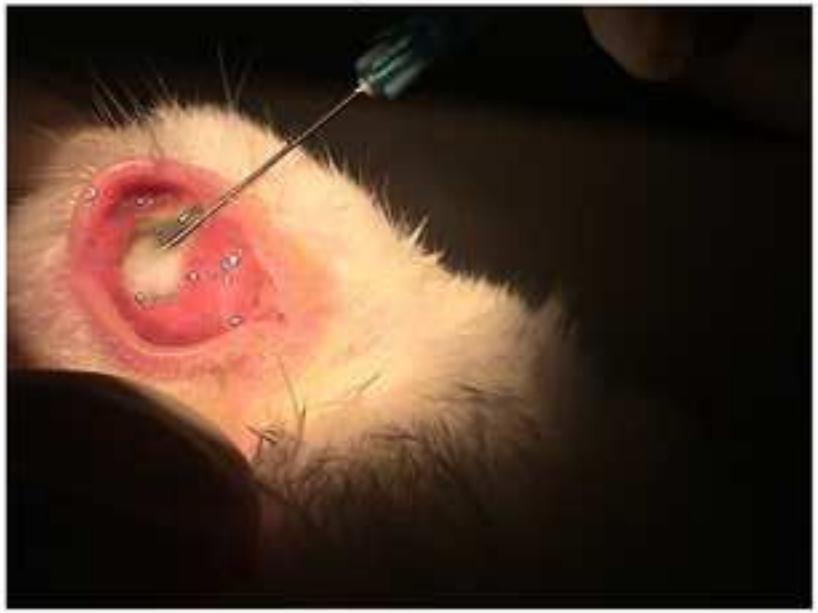


Figure 1B

Figure 1C

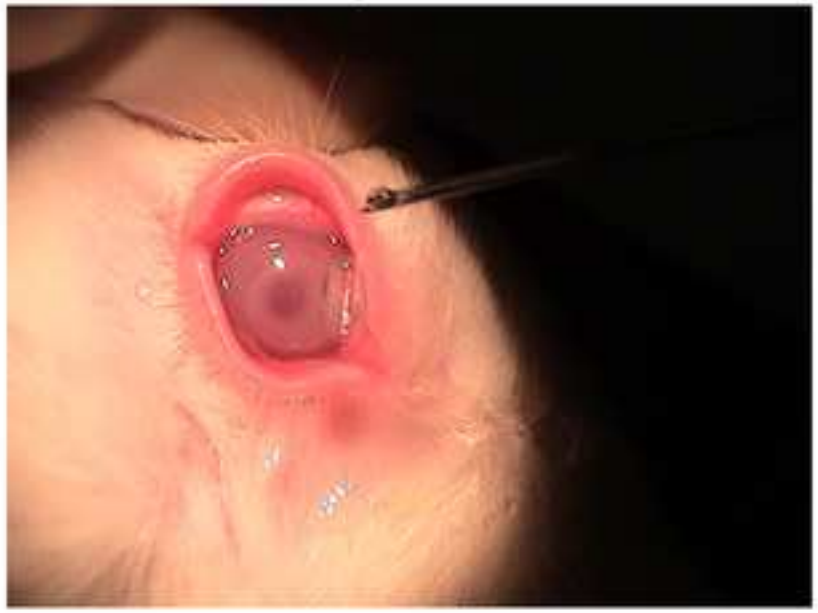
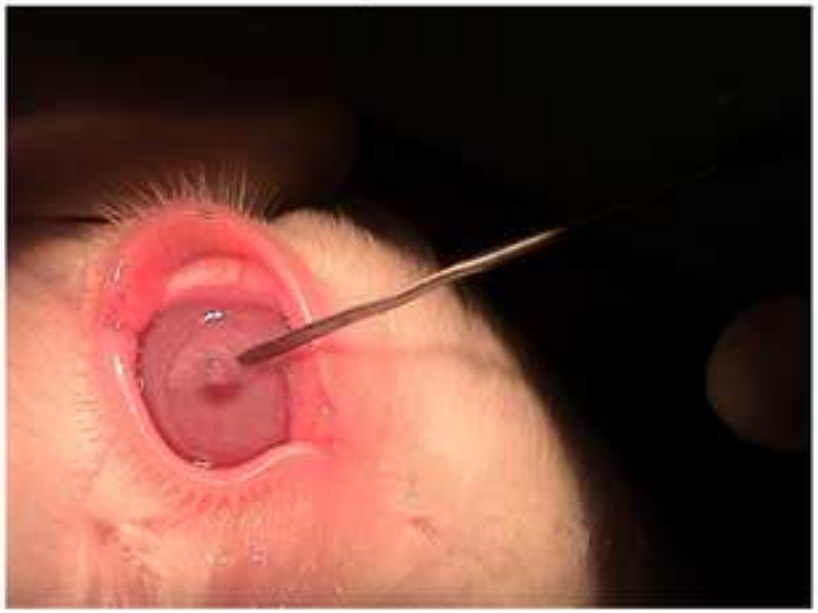


Figure 1D



Figure

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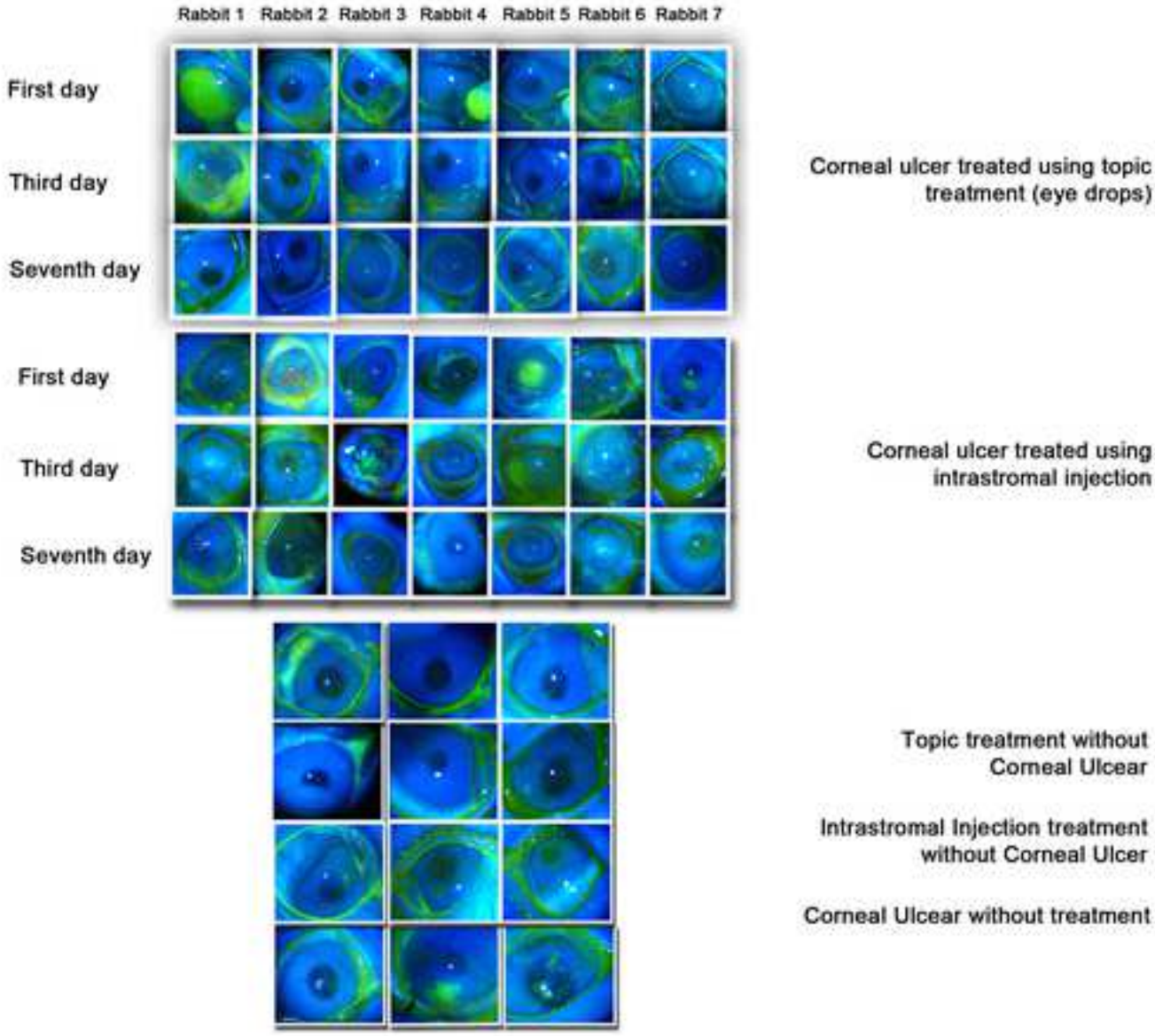
# Figure 2



Figure

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# Figure 3

## Group A

**Corneal Ulcer from rabbits treated using intrastromal injection**
Fibroblast proliferation, increased blood vesses, as well as inflammatory cells were observed below epithelium (Hematoxilin/Eosin, 100X)

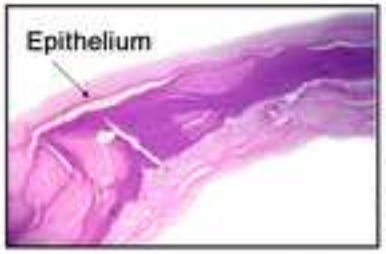
**Corneal Ulcer from rabbits treated using intrastromal injection**
Epithelium shows edema from the basal to middle layer. In stromal layer vessels with erythrocytes, fibroblast proliferation and scarce inflammatory infiltration was observed (Hematoxilin/Eosin, 40X)

## Group B

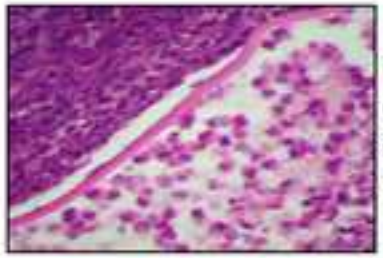
**Corneal Ulcer from rabbits treated using topic treatment (eye drops)**
Epithelium shows scarce edema and intrastromal layer shows increased fibroblasts (Hematoxilin/Eosin, 100X)

**Corneal Ulcer from rabbits treated using topic treatment (eye drops)**
Epithelium shows normal characteristics, with fibroblasts proliferation and scarce inflammatory cells (lymphocytes) (Hematoxilin/Eosin, 40X)

## Group C



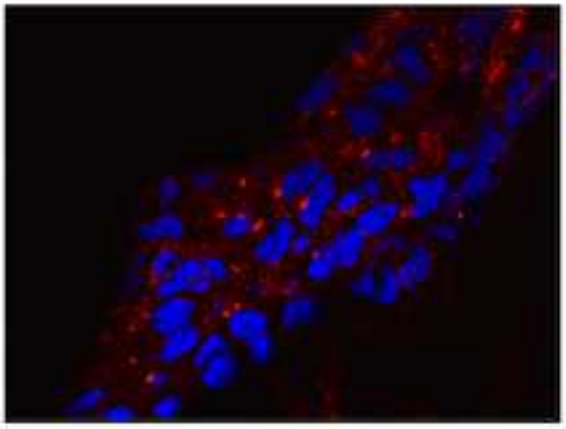

**Control.** Hisrological analysis of ulcerated comea Superficial epithelium shows necrotic corneal lamels. Fibrinoid maretial and inflammatory infiltrate was observed in the rear face of Descement (Hematoxilin/Eosin, 40X)



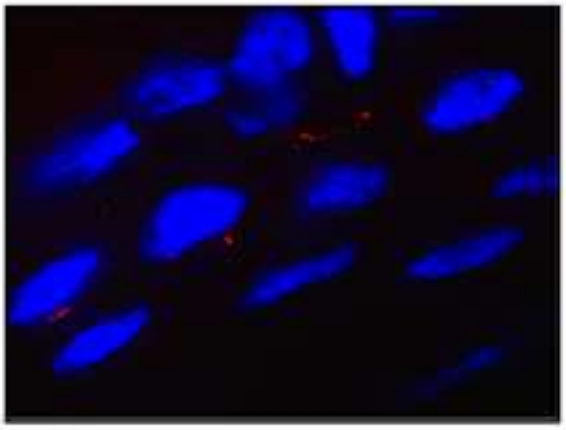
**Control.** Descemet membrane with remains of deep intrastromal tissue is observed. Also, inflammatory infiltrate is shown in the hipopion (Hematoxilin/ Eosin, 400X)

**Control.** Atrophic epithelium with loss of continuity in observed in the ulcer .Blood vessels and chronic inflammatory infiltrate is also shown (Hematoxilin/Eosin, 100X)

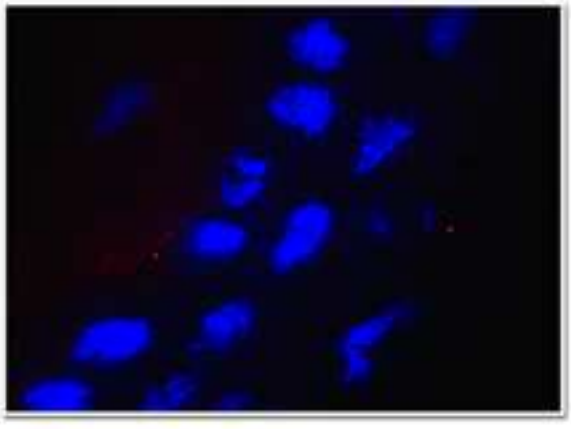
# Figure 4



A. Corneal ulceration without treatment



B. Corneal ulceration with stromal treatment



C. Corneal ulceration with topic treatment

Blue=Nuclei. Images acquired using ELYRA microscopy at 100x
a-SMA (actin of smooth muscle, in red)