·Basic Research·

Corneal collagen cross-linking and liposomal amphotericin B combination therapy for fungal keratitis in rabbits

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Abstract

• **AIM: To observe the therapeutic effect of corneal collagen cross-linking (CXL) in combination with liposomal amphotericin B in fungal corneal ulcers.**

• **METHODS: New Zealand rabbits were induced fungal corneal ulcers by scratching and randomly divided into 3 groups, *i.e.* control, treated with CXL, and combined therapy of CXL with 0.25% liposomal amphotericin B (n= 5 each). The corneal lesions were documented with slit-lamp and confocal microscopy on 3, 7, 14, 21 and 28d after treatment. The corneas were examined with transmission electron microscopy (TEM) at 4wk.**

• **RESULTS: A rabbit corneal ulcer model of *Fusarium* was successfully established. The corneal epithelium defect areas in the two treatment groups were smaller than that in the control group on 3, 7, 14 and 21d (P< 0.05). The corneal epithelium defect areas of the combined group was smaller than that of the CXL group (P<0.05) on 7 and 14d, but there were no statistical differences on 3, 21 and 28d. The corneal epithelium defects of the two treatment groups have been healed by day 21. The corneal epithelium defects of the control group were healed on 28d. The diameters of the corneal collagen fiber bundles (42.960±7.383 nm in the CXL group and 37.040±4.160 nm in the combined group) were thicker than that of the control group (24.900±1.868 nm), but there was no difference between the two treatment groups. Some corneal collagen fiber bundles were distorted and with irregular arrangement, a large number of fibroblasts could be seen among them but no inflammatory cells in both treatment groups.**

• **CONCLUSION: CXL combined with liposomal amphotericin B have beneficial effects on fungal corneal ulcers. The combined therapy could alleviate corneal inflammattions, accelerate corneal repair, and shorten the course of disease.**

• **KEYWORDS:** corneal collagen cross-linking; liposomal amphotericin B; fungal keratitis; confocal microscope; rabbit
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INTRODUCTION

Fungal keratitis is a type of infectious keratitis that has a high incidence in China [1]. There are two types of pathogens: yeast and molds. Yeast infection is often seen among patients with ocular diseases [2], while infection with molds (often called filamentous fungi) is commonly seen among patients with trauma to the eye or who wear corneal contact lenses; the incidence of mold infections is higher than that of yeast. *Fusarium* is one common mold causing fungal keratitis in most parts of China. The limited categories and low permeability of antifungal drugs make the treatment of fungal keratitis difficult, and outcomes remain poor.

Corneal collagen cross-linking (CXL) of corneal stromal collagen fibers, induced by ultraviolet light A (UVA) and riboflavin (vitamin B_2), can increase the hardness of the cornea and enhance the mechanical and biological chemical stability of the corneal stroma. CXL has been shown to have remarkable curative effects in patients with keratoconus and corneal ectasia [3-5], and has also shown efficacy in the treatment of refractory infectious keratitis after the failure of conventional antibiotic therapy [6-12]. Furthermore, we have previously reported clear therapeutic effects with CXL in animal experiments of corneal ulcers caused by *Fusarium*[13]. Overall, CXL can accelerate corneal epithelium repair, prevent corneal ulcers, and even preserve useful vision.

Amphotericin B is a type of alkene antifungal drug that has become an important therapy in clinics. In 2010, Sauer *et al*[14] reported that the antimicrobial properties of riboflavin/UVA (365 nm) against *Candida albicans*, *Fusarium* species, and *Aspergillus fumigatus* were improved by previous treatment with amphotericin B *in vitro*.

The aim of the current study was to observe the therapeutic effects of CXL in combination with liposomal amphotericin B in a rabbit model of fungal keratitis. We assessed the therapeutic effect of this combination by determining the clinical course of disease, regrowth of the corneal epithelium, and expression under a confocal microscope, and investigated the mechanism of action of the combination by observing changes in corneal collagen fibers under transmission electron microscopy (TEM).

MATERIALS AND METHODS

Experimental Animals Fifteen healthy, 8-week-old New Zealand white rabbits (weight 1.5-2.0 kg) were selected from the Medical Animal Center of Xi'an Jiaotong University. Each animal had a corneal thickness of greater than 500 μm, as assessed by anterior segment optical coherence tomography. Approval for this study was obtained from the Ethics Committee of the First Hospital of Xi'an. All animal experiments adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research.

Animal Model A fungal corneal ulcer was induced in each rabbit using corneal scratching and a decellularized corneal covering, as reported previously [13]. In brief, dry-stored heterogeneous cell-free cornea (Shaanxi Ophthalmological Institute) was rehydrated for at least 10min; 0.4 mL sumianxin II (a compound anesthetic agent that consists of xylidinothiazoline, EDTA, and the hydrochloric acids hydrogen etorphine and haloperidol; Animal Pharmaceutical Co., Ltd., Dunhua, Jilin Province, China) was used for anesthesia. Following an intramuscular injection of anesthetic, a drop of oxybuprocaine hydrochloride (Santen Pharmaceutical Co., Ltd., China) was twice added to each rabbit's right conjunctival sac at 5min intervals. Sterile drapes were spread and the eyelid was opened using an eye speculum. A trephine was used to create a 7.5 mm diameter crease in the center of the cornea, following which the central corneal epithelium was shaved and the central area was scratched with a sterile needle, and *Fusarium* liquid (Shaanxi Ophthalmological Institute) was smeared on this area. The heterogeneous cell-free cornea was clipped to give a 7.5 mm diameter after rehydration, and sutured onto the central cornea of the experimental rabbits using a 10/0 nylon line. *Fusarium* liquid 0.1 mL was injected under the heterogeneous cell-free cornea, and the eyelid was sutured with black silk. The eyelid sutures were removed after 1wk, at which time the heterogeneous cell-free cornea was also removed and *Fusarium* infection was examined using a slit lamp, anterior segment camera, and confocal microscopy.

Grouping and Examinations Five rabbits were randomly selected to form the control group, while the other 10 rabbits were randomized to receive either CXL or combination therapy (n=5 in each group). The corneal lesion was examined daily using a slit lamp, and its diameter was measured using calipers; meanwhile, cornea edema and stromal infiltration were observed on days 3, 7, 14, 21 and 28. At the same timepoints, the condition of the ulcer was examined using an anterior segment camera and the *Fusarium* infection was examined using confocal microscopy.

Corneal Collagen Cross-linking Rabbits in the CXL and combination groups were given an intramuscular injection of 0.4 mL sumianxin II for anesthesia. Following this, oxybuprocaine hydrochloride eye drops were twice applied to the right conjunctival sac at 5min intervals. Then, 0.1% riboflavin (Veni Vidi, HAUS Mdizinprodukte GmbH, Kiel, Germany) was applied as drops after being dissolved in 20% dextran, for a duration of 30min at 5min intervals. At the same time, entry of riboflavin into the anterior chamber was confirmed by observing a yellow dye in the aqueous humor under a slit lamp with a cobalt blue filter. The rabbit was then put on the rabbit clip, the eyelid was opened using an eye speculum, and a cross-linking instrument (IROC AG, Zurich, Switzerland) was used to irradiate the eye for 10min (UV light wavelength 370±5 nm, radiation degree 9.7-9.8 mW/cm^2), with the beam diameter determined by the lesion size.

Eye Drops with 0.25% Liposomal Amphotericin B Under sterile conditions, 4 mL sterile water was extracted using a 5 mL sterile syringe and injected into a bottle containing 10 mg liposomal amphotericin B powder (Shanghai New Pioneer Pharmaceutical Co., Ltd., China). The mixture was well shaken, and then transferred to an empty chloromycetin bottle and stored in a refrigerator at 4℃. Drops were applied to the rabbits in the combination group according to the following schedule: once every 15min for the first hour; once every 30min after 1h; once every hour after 24h; once every 2h after 48h; and then continuously.

Checking by Confocal Microscopy Oxybuprocaine hydrochloride eye drops were twice applied to the right conjunctival sac at 5min intervals. The sequence numbers of the rabbits were entered into a computer connected to confocal microscopy (HRT-III; Heidelberg Company). Carbomer eye drops (10 g; from Dr. Gerhard Mann, Chem.-Pharm. Fabrik GmbH) were placed on the microscope probe, followed by a disposable sterile cap. Each rabbit's eyelid was opened using an eye speculum and an assistant fixed the rabbit's head in position. The imaging plane was adjusted by

Table 1 The diameter of corneal epithelium defect mm

Group	3d	7d	14d	21d	28d
Control	5.720±1.686	5.420±0.512	3.560±0.730	1.020±0.870	0.000±0.000
Combination	3.413±2.506	3.480±0.999	0.540±0.555	0.000±0.000	0.000±0.000
CXL	4.173±2.275	4.780±0.845	1.400±0.946	0.000±0.000	0.000±0.000

changing the contact between the probe and the corneal lesion using the handle on the host, and an image of each layer of the cornea was taken using a charge-coupled device (CCD) camera. Images were displayed on a computer screen and saved to the computer.

Specimen Preparation and Management All of the rabbits were sacrificed after 4wk treatment. The cornea tissue was sheared and examined under TEM.

Statistical Analysis Data are shown as means ±standard deviation. Differences between means were calculated using variance analysis of multiple comparisons using SPSS 13.0 statistical software. P values of less than 0.05 were considered to be statistically significant.

RESULTS

A rabbit corneal ulcer model of *Fusarium* was successfully achieved using corneal scratching and a decellularized corneal covering (Figure 1). The corneal lesions were gray, with opacity and no clear boundary, and satellite lesions, epithelial defects, a dry surface, edema matrix, and conjunctival congestion were all observed. The mean diameter of the corneal epithelium defect was 7.5 mm, with no significant difference between the three groups prior to treatment. Corneal melting was present in localized lesions. Confocal microscopy identified a large number of short-rod, branch-like hyphae, septate hyphae, and activated stromal cells.

On day 3 of treatment, rabbits in the two treatment groups exhibited a shrunken corneal epithelium defect range compared with the control group (both P<0.05), with no statistical difference between the treatment groups (P>0.05) (Table 1). The corneal stromas of all experimental rabbits showed edema, with a large number of hyphae in the shallow stroma.

On day 7 of treatment, the corneal epithelium defects were even further shrunken in the two treatment groups (Figure 2), and were much smaller compared with the control group (both P <0.05) (Table 1). Meanwhile, the area of the corneal epithelium defect was much smaller in the combination-therapy group than in the CXL group (P <0.05) (Table 1). Corneal edema and stromal infiltration were also significantly reduced in the combination-therapy group. The number and density of mycelia were all decreased in the two treatment groups (Figure 2), while more hyphae were observed in the corneal lesion area in the control group. New blood vessels were seen to be sprouting around the corneal lesion in the two treatment groups.

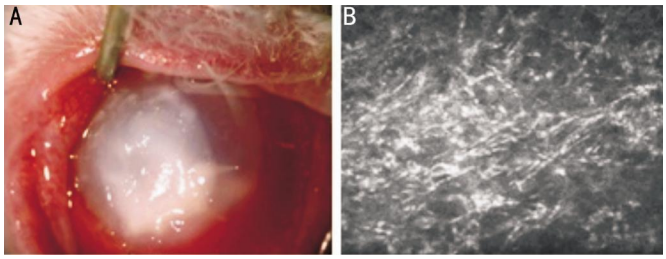

Figure 1 The rabbit corneal ulcer model of *Fusarium* A: The manifestation of corneal ulcer by anterior segment camera; B: The manifestation of hyphae by confocal microscopy.

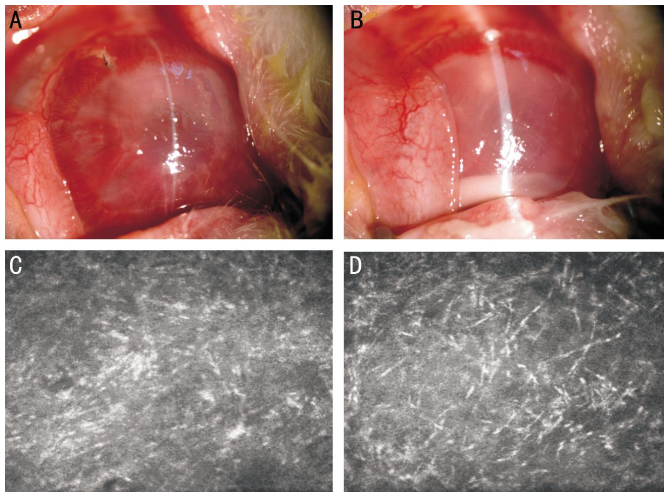

Figure 2 On day 7, the corneal epithelium defect of the two treatment groups and the hyphae manifestation by confocal microscopy A: The corneal epithelium defect area of the combination group; B: The corneal epithelium defect area of the CXL group; C: The hyphae manifestation of the combination group by confocal microscopy; D: The hyphae manifestation of the CXL group by confocal microscopy.

On day 14 of treatment, the corneal epithelium defect in the two treatment groups continued to shrink. The range of the defect was still less in the two treatment groups than in the control group (both P <0.05), and the range of the defect remained lower in the combination-therapy group compared with the CXL group (P <0.05) (Table 1). In the combination-therapy group, the peripheral new blood vessels extended to the center of the cornea, the corneal stroma showed mild edema, and the hyphae were inactive (Figure 3). The CXL group showed a small number of active hyphae in the cornea (Figure 3), while hyphae remained visible in the control group.

On day 21 of treatment, the corneal epithelium of the control group was still absent, but complete epithelialization had occurred in the two treatment groups, and only some colored

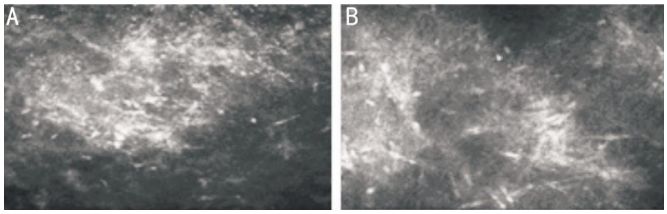

Figure 3 On day 14, the corneal ulcer manifestation of the treatment groups by confocal microscopy A: The manifestation of the combination treatment group; B: The manifestation of the CXL group.

dots were seen. Corneal neovascularization was sparse in the combination-therapy group, and there was no edema in the corneal stroma and no obvious hyphae, but scar tissue had formed (Figure 4). Corneal neovascularization in the CXL group was dense (Figure 4), edema was obvious in the stroma, and hyphae showed disintegration and appeared highly reflective. At the same timepoint, there were inactive hyphae in the control group.

On day 28 of treatment, each group showed a complete corneal epithelium. In particular, the control group was self-healing, with no stromal edema or hyphae and formation of a scar (Figure 5).

Transmission Electron Microscopy TEM showed that the control group had plenty of dense collagen fiber bundles under the cornea basement membrane. The collagen fibers were crisscrossed and in neat rows, with a few fibroblasts seen between them. There were also plenty of dense collagen fiber bundles under the cornea basement membrane in the two treatment groups, although some were distorted and had a disordered arrangement. In addition, there were a large number of fibroblasts between the collagen fibers, and the cell volume was larger than that in the control group and the shape was irregular. Furthermore, the electron density was higher, protrusions were longer, and the rough endoplasmic reticulum and mitochondria were well developed.

The corneal collagen fiber diameter was 23-27 nm (average 24.900±1.868 nm) in the control group, 32-50 nm (average 42.960 ±7.383 nm) in the CXL group, and 32-43 nm (average 37.040 ±4.160 nm) in the combination-therapy group (Figure 6). The collagen fibers were thicker in the two treatment groups compared with the control group (P<0.05), but there was no statistical difference between the treatment groups (P>0.05).

DISCUSSION

Fungal keratitis can be a devastating infectious corneal disease. It usually has a slow onset with patients experiencing few subjective symptoms, which often leads to a late diagnosis. The limited categories and low permeability of antifungal drugs makes the treatment of fungal keratitis difficult, with poor outcomes for patients. Patients with serious infections can be treated with penetrating

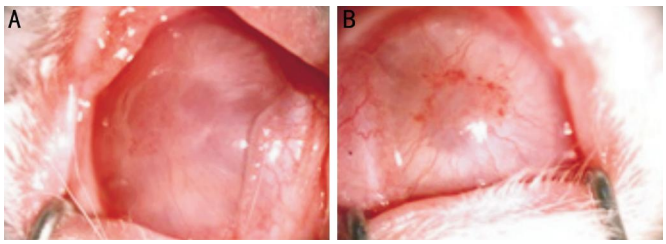

Figure 4 On day 21, the manifestation after epithelialization of the treatment groups A: The cornea of the combination treatment group; B: The cornea of the CXL group.

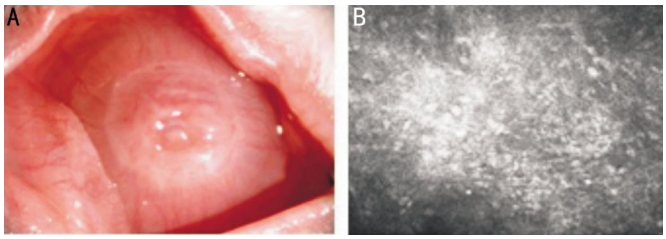

Figure 5 On day 28, the manifestation after healing of the corneal ulcer in the control group A: The cornea of the control group; B: The manifestation by confocal microscopy.

keratoplasty, but insufficient corneal materials are available to meet the needs of the large number of patients. Therefore, fungal keratitis eventually leads to eye evisceration in many patients, which is not only accompanied by great pain, but also seriously affects patients' quality of life. Therefore, safe and effective new therapies are needed for the treatment of this condition.

The effective component of liposomal amphotericin B is amphotericin B. Liposomes can increase the affinity between amphotericin B and sterols in the fungal cell and, at the same time, reduce the affinity of the mammalian cell membrane to cholesterol. Therefore, they not only increase antifungal activity, but also reduce damage to the host tissues. Liposomal amphotericin B has become an important antifungal drug in clinics.

The basic principle of CXL is using UV light of 370 nm wavelength to illuminate the sensitizer riboflavin and generate reactive oxygen species, mainly composed of singlet oxygen. The reactive oxygen species interact with various molecules, and then induce chemical cross-linking between collagen fiber aminos (II photochemical reaction) and form covalent bonds between the fibers [15-16]. The biomechanical properties of the cornea depend on collagen, collagen fiber bundles, and their spatial structure and so, after cross-linking, the mechanical strength of the cornea and its ability to resist keratectasia are increased. Studies have shown that the stabilized biological chemistry of the cornea following CXL results from a change in the tertiary structure between collagen fibers. Proteolytic enzymes are prevented from binding to their specific loci after CXL, which can thus inhibit collagenolysis. Various authors have attempted to use CXL as an adjuvant treatment or even as the sole treatment

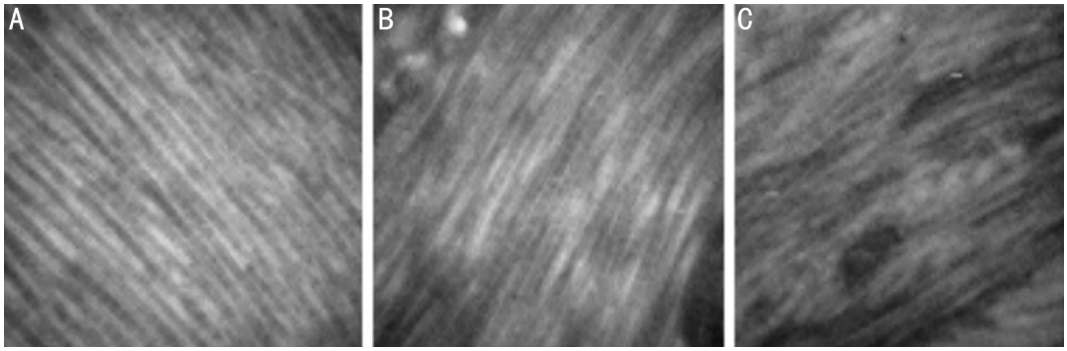

Figure 6 The corneal collagen fiber diameter of the three groups A: The corneal collagen fiber diameter of the control group was 23-27 nm (TEM 100 000×); B: The corneal collagen fiber diameter of the CXL group was 32-50 nm (TEM 100 000×); C: The corneal collagen fiber diameter of the combination-therapy group was 32-43 nm (TEM 100 000×).

for infectious corneal ulcers. The current study used an irradiation protocol of 9.7-9.8 mW/cm^2 for 10min, which Tabibian *et al* [11] have previously reported to be as effective as conventional irradiation with 3 mW/cm^2 for 30min.

In the current study, a rabbit corneal ulcer model of *Fusarium* was successfully achieved using corneal scratching and a decellularized corneal covering. This method not only provided the appropriate temperature and humidity for fungal growth, but also allowed the retention of a sufficient quantity of bacterial liquid and avoided washing with tears, leading to a high rate of mold growth. Corneal melting was controlled and complete epithelialization was achieved in all experimental rabbits without perforation; the basic disease course lasted 4wk in the control group, but was reduced to 3wk in the two treatment groups. The speed of healing was fastest in the combination-therapy group, and the therapeutic effects of CXL in combination with liposomal amphotericin B were remarkable. TEM showed that the corneal collagen fibers had a greater diameter in the two treatment groups compared with the control group, with no statistical difference between the treatment groups, and a large number of fibroblasts were seen between the collagen fibers in these groups.

The combined action of CXL and liposomal amphotericin B might work through the following mechanisms. First, the combination of UVA irradiation and liposomal amphotericin B might enhance direct damage to the pathogenic microorganism, accelerating the death of the pathogen, inhibiting fungal growth, and reducing inflammation, and therefore shortening the course of the disease. However, the exact mechanism behind this combined action is unclear. Second, the photosensitizer riboflavin, irradiated by UVA in the cornea, could reinforce the corneal collagen and enlarge the diameter of the corneal collagen fibers, and therefore improve the resistance of the cornea to damage and fungal invasion. Third, CXL might stimulate the proliferation of fibroblasts and then accelerate corneal repair. Fourth, the ability of the cornea to resist enzymatic digestion is significantly strengthened after treatment with CXL[17], which might help the cornea to avoid perforation. Finally, the rabbit's strong ability to repair itself and fast growth of new blood vessels means ulcers have a tendency to self-heal.

Although some experimental and clinical studies have preliminarily confirmed the curative effect of CXL, its application has certain restrictions [15,18]. First, the corneal thickness must be at least 400 nm. Second, the UV wavelength and energy must be below the threshold that would cause damage to the eye.

In this study, we found that CXL was safe and efficacious in treating fungal keratitis in a rabbit model of the condition[13]. The combination of CXL and liposomal amphotericin B was associated with a significantly reduced inflammatory reaction, faster corneal repair, a shorter duration of symptoms, and reduced new blood vessel growth. These findings are of great significance in helping patients to retain useful vision and have strong clinical value. CXL represents a cost-efficient, viable, and safe therapeutic option for the treatment of severe infectious corneal ulcers. Based on current evidence, however, the role of CXL in infectious keratitis remains unclear, and its long-term therapeutic efficacy, safety, and effect on the physical and chemical properties of the cornea all require further research.

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