

Experimental study on the treatment of rabbit corneal melting after alkali burn with Collagen cross-linking

Xiao-Wei Gao¹, Xu-Dong Zhao², Wen-Jing Li¹, Xin Zhou¹, Ying Liu²

¹Ophthalmic Center, No.474 Hospital of Chinese PLA, Urumchi 830013, Xinjiang Uygur Autonomous Region, China

²The Medical College of Shihezi University, Shihezi 832002, Xinjiang Uygur Autonomous Region, China

Correspondence to: Xiao-Wei Gao. The Medical College of Shihezi University, Shihezi 832002, Xinjiang Uygur Autonomous Region, China. gxwgaowx@263.net

Received: 2011-12-16 Accepted: 2012-01-30

Abstract

• **AIM:** To evaluate the effect of Collagen cross-linking on the prevention of melting in rabbit corneas after alkali burn.

• **METHODS:** Twenty New Zealand white rabbits were randomly divided into model control group and collagen cross-linking treatment group. The second group of rabbits received collagen cross linked treatment. Both groups were applied with antibiotic eye drops to prevent infection. The corneas were evaluated for melting, opacity, pathological and immunohistochemistry, record the changes when 28 days after the animals were killed.

• **RESULTS:** In the control group, 6 out of 8 rabbits showed corneal melting after injury (14± 4) days, while two corneal perforated. In collagen cross-linking treatment group, one rabbit showed corneal melting after injury 23 days, without corneal perforation; corneal dissolution rate between the two groups was significantly different ($P < 0.05$). Pathological examination suggested that in the treatment group, mild corneal edema, mild damage to collagen fibers, inflammatory cell infiltration was significantly less than the control group. Immunohistochemistry showed that corneal collagen fibers arranged in neat rows in the control group.

• **CONCLUSION:** Collagen cross-linking treatment not only can prevent and delay the corneal melting after alkali burn, but also can reduce the destruction of corneal collagen fibers and infiltration of inflammatory cells in the corneal tissue.

• **KEYWORDS:** collagen cross-linking; corneal alkali burn; corneal melting; rabbit

DOI:10.3980/j.issn.2222-3959.2012.02.06

Gao XW, Zhao XD, Li WJ, Zhou X, Liu Y. Experimental study on the treatment of rabbit corneal melting after alkali burn with Collagen cross-linking. *Int J Ophthalmol* 2012;5(2):147-150

INTRODUCTION

Corneal melting after alkali burn has been one of clinical Ophthalmology tricky problems ^[1], which can cause corneal perforation and eventually cause permanent vision impairment. Various reasons for corneal melting include corneal alkali burns, immune factors, infections, and eye surgery, systemic diseases (rheumatoid arthritis) etc. Since its pathogenesis is not fully clear, there is no final solution for this in clinical practice. Present study suggests that the mechanism of corneal melting associated with the destruction of the corneal collagenase, can reduce the antigen produced by the cornea, thereby reducing leukocyte aggregation, breaking the vicious cycle, to achieve the purpose of the treatment of corneal melting. Corneal collagen cross-linking is a way used to treat keratoconus in clinical practice. *In vitro* experiments confirmed that after the treatment, can enhance the tolerance of a variety of enzymes, has anti-bacterial, and enhance the hardness of the cornea ^[2], but the effect of this treatment to melting of the cornea after alkali burn not yet reported. In this study, rabbit corneal melting following alkali burn animal model was produced the effect of corneal cross-linking technique for the treatment of corneal melting was assessed.

MATERIALS AND METHODS

Materials Twenty New Zealand white rabbits, weighing between 1.8-2.2kg, of both sexes, were supplied by the Experimental Animal Center of Xinjiang Medical University. The animals and experimental conditions followed laboratory animal regulations of State Science and Technology Commission. Animals were randomly divided into two groups, group A (model control group) group B (collagen cross-linking treatment group) with 10 rabbits in each group. OPTO X-Link-Corneal Crosslinking system (OPTO ELETRONICA S.A, BRAZIL). Preparation of riboflavin solution: the People's Liberation Army 474 Hospital Pharmacy under sterile conditions, a concentration of 0.1% riboflavin eye drops using 20% dextran T500 (Pharmacia Co., Ltd) 2g in 10mL saline, riboflavin 10mg (Japan Co., Ltd) were prepared and kept in a sealed bottle at 4°C for use. Collagen antibody (Santa Co., Ltd); SP kit and Diaminobenzidine (DAB) chromogenic kit (Beijing Kang Century Biotech Co., Ltd.).

Table 1 Comparison of corneal melting and perforation between two groups

Group	Cases	Corneal melting		Corneal perforation		Corneal melting score	Corneal opacity score
		Cases	time	cases	time		
A	10	6	14±4	2	23±2	2	4
B	10	1	23	0	0	0.1	3

Methods

Preparation of animal model of alkali burn For general anesthesia, 2mL of 50mg/kg ketamine and 0.5mL of 10mg/kg chlorpromazine were injected intramuscularly into the rabbits' hind legs. 0.4% Ao Buka was used for ocular surface anesthesia. 8mm diameter filter paper immersed in 1 mol/L Sodium hydroxide solution 60 seconds, and then put on the center of animal right eye for 30 seconds. The eye was washed immediately with Saline solution for 2 minutes. Clear disc-shaped white burn area was seen on the cornea. This was moderate alkali burn according to Hughes indexing method.

Cross-linking treatment After the model, riboflavin photosensitizer solution containing 0.1% riboflavin-5-phosphate and 20% dextran T-500 was dropped onto the cornea every 5 minutes, initiating 30 minutes before the irradiation. Collagen cross-linking treatment for 30 minutes (OPTO X-Link-Corneal Crosslinking system, OPTO ELETRONICA S.A., BRAZIL) was given using the Cross-linking system (wavelength is 370nm; illumination is 3.0mW/cm²; irradiation diameter was 8mm; 0.1% riboflavin eye drops were dropped every 3 minutes ×10 times) was applied 45mm above the cornea.

Corneal opacity and corneal melting Score After Alkali burns modeling, using slit-lamp microscope corneal melting score as per Pfister *et al*^[3] and corneal opacity score as per Holland *et al*^[4] were recorded on the 3, 7, 14, 21, 28 days. Five rabbits were selected in each group randomly. Under general anaesthesia, eyes were enucleated and corneas were sent for histopathology. HE staining was done to observe pathological changes in corneal tissue. Randomly selected slices without staining, according to the instruction of SP kit, were used for observation of corneal collagen type I levels of expression. Corneal tissues with brown-yellow granules were taken as positive. Colour images of selected corneal tissue under low magnification were taken using Olympus BX41 computer image analysis system.

Statistical Analysis Statistical analysis was performed using the SPSS 17.0 statistical software. All data were expressed as the mean ± standard deviation (SD). The rate was compared with Chi-square test. Compare ranked data with rank sum test, *P* < 0.05 was considered statistically significant.

RESULTS

All animals had no infections and no accidental death. After

corneal alkali burns there was a clear boundary of edema on the cornea; then the edema reduced gradually. Treatment group and control group during treatment, repeated corneal epithelial hyperplasia, exfoliation. Six cases of corneal melting occurred in A group, one cases of corneal melting occurred in B group, the difference between the two groups was statistically significant (*P* = 0.029 < 0.05). Corneal melting in Group A occurred 14 days after injury (range 9-20 days); corneal melting in Group B occurred after injury 23 days (range 23 days); the difference between the two groups was statistically significant (*P* = 0.253 > 0.05) (Table 1). Corneal perforation occurred 2 cases in Group A on 21st and 25th day. There was no corneal perforation in Group B, the difference between the two groups was not significant (*P* = 0.237 > 0.05) (Table 1). At 28 day after modeling, the corneal melt average score in Group A was 2 points and in group B was 0.3 points; compare the clinical scores between the two groups with rank sum test, the difference was significant (*P* = 0.035 < 0.05) (Table 1). Corneal opacity between the two groups was not different after 5 days of alkali burns. Corneal opacity in group A began to increase after 9 days of injury, until the cornea completely became opaque. Group B corneal opacity started after 7 days of injury and reduced to the end of treatment. Corneal opacity was always less than in group A. The corneal opacity score between the two groups after burn 28 days with rank sum test, was significant different (*P* = 0.002 < 0.05, Table 1).

Histopathological Examination (HE staining) Normal control group: the corneal layers were clear, the corneal stroma arranged rules. The control group-corneal edema severe, epithelial proliferation significantly, stroma damaged severely, collagen fibers degeneration, disorganized. There was eosinophilic matter accumulation in corneal layer, fiber proliferation on the corneal surface; corneal stroma showed a large number of particles of leukocyte infiltration. Treatment group-corneal epithelial hyperplasia, collagen destruction in the anterior cornea stroma, lighter than control group, leukocyte infiltration in the anterior cornea stroma, less than the control group, the deep corneal stroma collagen fibers arranged regularly, less corneal swelling (Figure 1).

Immunohistochemical Immunohistochemical staining of collagen type I: the control group-the corneal stroma arranged regularly clear hierarchy. The control group: degeneration of collagen fibers, stroma layer disordered

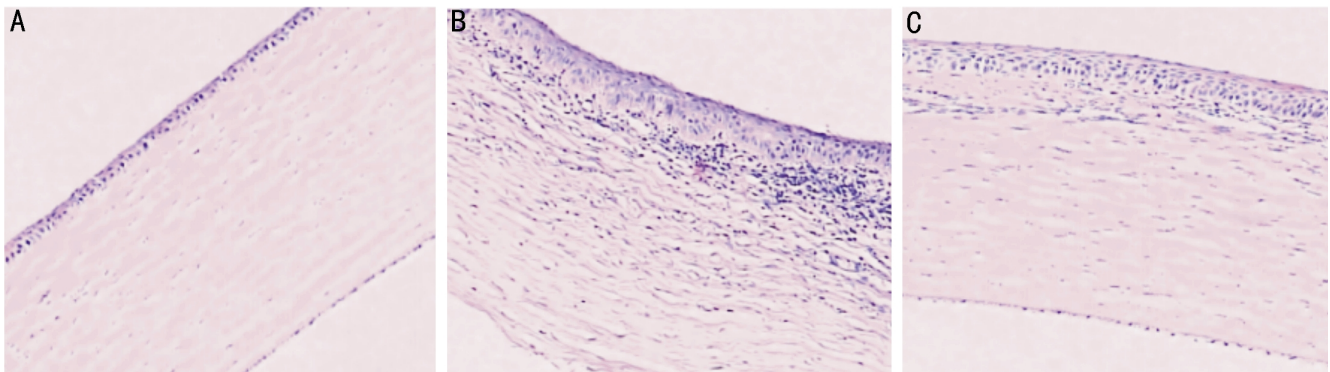


Figure 1 Rabbit corneal tissue HE staining A: normal control group, hierarchical layers of the cornea clear corneal stroma arranged rules. (HE×100); B: model group, corneal edema and epithelial hyperplasia+, stroma severely damaged, disorganized, and corneal stroma shows a large number of particles of leukocyte infiltration (HE×100); C: Treatment group, corneal epithelial hyperplasia+, visible damage before the matrix layer, lighter, front visible particles leukocyte infiltration than the control group, the deep corneal stroma fibers rules (HE×100).

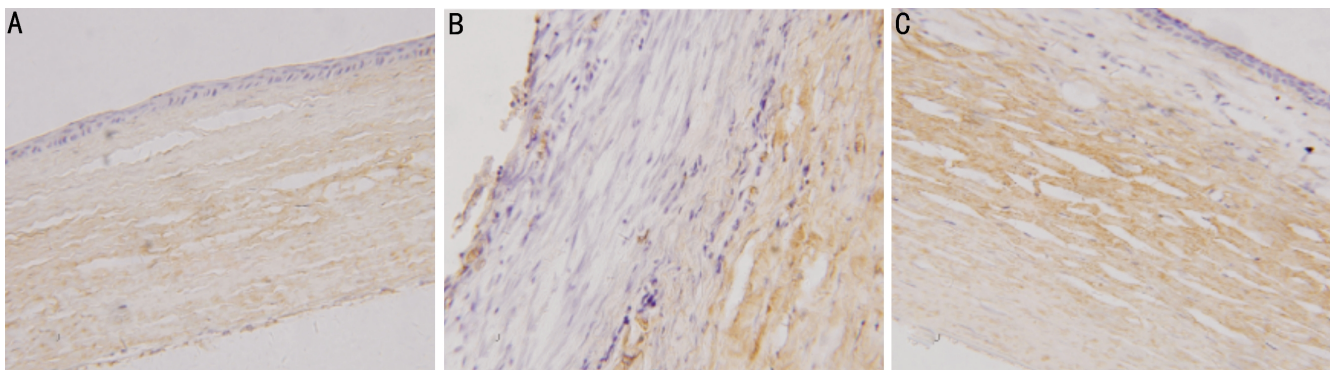


Figure 2 Expression of type I collagen in rabbit corneal A: Normal control group expression in corneal stroma, arrange the rules (×100); B: Model control group expressions, corneal stromal edema, arranged collagen disorder, levels are not clear (×100); C: Treat group stroma collagen fibers destruction in the anterior cornea, deep-rule (×100).

significantly in full-thickness in the cornea. Treatment group-stroma collagen fibers destruction in the anterior cornea; posterior corneal collagen fibers arranged in neat rows (Figure 2).

DISCUSSION

Corneal melting is caused by a variety of risk factors of non-infectious corneal ulcers such as immune factors, collagen vascular disease and systemic disease, and it can occur in a variety of corneal diseases such as Mooren ulcer, corneal chemical burns (especially alkali burns), eye injury, eye Surgery and keratitis, its exact pathogenesis is not clear^[5]. Present study suggests that the main mechanism of corneal melting is that a large number of alkali burn polymorphonuclear leukocyte infiltration, collagenase and matrix metalloproteinase continuous digestion of corneal collagen^[6] and oxygen free radicals damage to the corneal cell toxicity^[7]. Involved in the immune system: immune inflammatory cells^[8] (including polymorphonuclear leukocytes, lymphocytes, Lange Hansi cells) and immune molecules^[9] (such as cytokines, adhesion molecules, chemokines, etc.) interact with each other to form complex of immune and inflammatory network, mediated corneal pathology.

The current symptomatic treatment drugs include^[10]: 1) anti-inflammatory treatment: tobramycin, ofloxacin etc; 2) collagenase inhibitors: EDTA-2Na, cysteine, matrix metalloproteinase inhibitors such as GM6001; 3) drugs which promote collagen synthesis and stromal cells: vitamin C, fibronectin; 4) leukocyte infiltration inhibitors: sodium citrate; 5) immunosuppressive agents such as dexamethasone. These treatments in vitro experiments have some effect, but the clinical effect is not clear.

Corneal cross-linking treatment with riboflavin as cross-linking agent: at 370nm wavelength of UV-A irradiation, riboflavin stimulates generation of reactive oxygen free radicals, which can form covalent bond between and within collagen fibers and a variety of molecular further more, enhance the hardness of the cornea^[11]. Ultra structural examination showed collagen fiber diameter increased after corneal cross-linking, with enhancement of corneal lamellar fiber connection between and within the cornea layer^[12]. This study evaluated the influence of the cross-linking treatment with riboflavin on the treatment of corneal melting through rabbit corneal melting model of moderate alkali burn. The results showed that corneal collagen cross-linking treatment can reduce the incidence of corneal melting after corneal alkali burn, also can reduce corneal opacity.

Histopathology examination showed that after corneal collagen cross-linking treatment corneal epithelial hyperplasia less than the control group; inflammatory cell infiltration, deep corneal damage was less.

Immunohistochemical staining showed that treatment of corneal collagen arranged in neat rows, light damage than the control group. Collagen cross-linking treatment of corneal alkali burn mechanism may have two reasons: (1) after corneal cross-linking the collagen fibers increased and the covalent bond reduces the ability of corneal melting; also reduces the melting product, breaking the vicious cycle of corneal alkali burns; (2) Corneal collagen cross-linking has effect on the anterior corneal stroma, the cornea can increase the tolerance of hydration and reduce corneal edema^[13,14]; thus, protects the deep corneal tissue. Therefore, reduces the inflammatory cell infiltration, collagen fibers arranged in neat rows. In vitro studies revealed that cornea after cross-linking soaked in pepsin, collagenase and trypsin solution, showed melting in the first 13 days, 14 days and 5 days respectively, while the untreated cornea showed melting in the first 6 days, 6 days, the first 2 days respectively. Following cross linking treatment, there is formation of new covalent bond between collagenous fibre, resulting in new molecule space structure, causing collagenase destruction to the corresponding molecular position spot. Thus, after crossing linking treatment cornea resists enzyme digestion for certain period of time^[2,15].

This experimental observation found that collagen cross-linking treatment can delay the occurrence of melting; reduce the damage to corneal collagen fibers, reduce inflammation of the corneal tissue cell infiltration.

In this study, there are some shortcomings, such as the observation time is shorter, a limited number of samples, the small number of corneal perforation which may influence statistical, analysis of the comparison of corneal melting time. Collagen cross-linking is a new treatment method for keratoconus, although the clinical report found no significant complications^[16], but the effect of collagen cross-linking of the cornea after alkali burn needs further study.

REFERENCES

- 1 Fini ME, Cook JR, Mohan R. Proteolytic mechanisms in corneal ulceration and repair. *Arch Dermatol Res*1998; 290(suppl): S12–S23
- 2 Spoerl E, Wollensak G, Seiler T. Increased resistance of cross-linked cornea against enzymatic digestion. *Curr Eye Res* 2004;29(1):35–40
- 3 Pfister RR, Paterson CA, Hayes SA. Topical ascorbate decreases the incidence of corneal ulceration after experimental alkali burns. *Invest Ophthalmol Vis Sci* 1978;17(10):1019–1024
- 4 Holland EJ, Chan CC, Wetzig RP, Palestine AG, Nussenblatt RB. Clinical and immunohistologic studies of corneal rejection in the rat penetrating keratoplasty model. *Cornea*1991;10(5):374–380
- 5 Sun BJ, Xu JT. The theoretical basis and clinical of Keratopathy. Beijing: Scientific and Technological Literature Publishing House. 1994:279–283
- 6 M. Elizabeth Fini, Jeffery R, Cook, Royce Mohan. Proteolytic mechanisms in corneal ulceration and repair. *Arch Dermatol Res* (1998) 290 (Suppl): S12–S23
- 7 Zhao Min, Chen Jia-Qi, Yang Pei-Zeng. Immunologic mechanism of Corneal Alkali Burn in Rabbits. *Chin J Ophthalmol*2000; 36(1):40–42
- 8 Kao WW, Zhu G, Benza R, Kao CWC, Ishizaki M, wander A H. Appearance of immune cells and expression of MHC- II DQ molecule by fibroblast in alkali burned corneas. *Cornea*1996; 15(4):397–408
- 9 Yuan J, Chen JQ, Liu ZG, Wang CZ, Zhou SY, Huang T, Xu LY. Study of immunology mechanism of alkali-burned corneas treated with IL-1ra. *Zhonghua Shiyan Yanke Zazhi*2007; 25(1):5–9
- 10 Burns FR, Gray RD, Paterson CA. Inhibition of alkali-induced corneal ulceration and perforation by a thiol peptide. *Invest Ophthalmol Vis Sci* 1990; 31(1):107–114
- 11 Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine cornea after riboflavin/ultraviolet-A-induced cross-linking. *J Cataract Refract Surg*2003; 29 (9):1780–1785
- 12 Meek KM, Tuft SJ, Huang Y, Gill PS, Hayes S, Newton RH, Bron AJ. Changes in collagen orientation and distribution in keratoconus corneas. *Invest Ophthalmol Vis Sci*2005; 46(6):1948–1956
- 13 Wollensak G, Aurich H, Wirbelauer C. Potential use of riboflavin/UVA cross linking in bullous keratopathy. *Ophthalmic Res*2009;41(2):114–117
- 14 Wollensak G, Aurich H, Pham D-T, Wirbelauer C. Hydration behavior of porcine cornea cross-linked with riboflavin and ultraviolet A. *J Cataract Refract Surg*2007; 33(3):516–521
- 15 Schilde. Enzymatic evidence of the depth dependence of stiffening on riboflavin/UVA treated corneas. *Dev Ophthalmologic*2008;105(2):165–169
- 16 Caporossi A, Mazzotta C, Baiocchi S, Caporossi T. Long-term results of riboflavin ultraviolet A corneal collagen cross-linking for keratoconus in Italy: the Siena Eye Cross Study. *Am J Sci*2010;149(4):585–593