Effect of 1- and 6-Hour-Delayed Corneal Collagen Cross-Linking on Corneal Healing in a Rabbit Alkali-Burn Model: Clinical and Histological Observations

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Purpose: To study the effect of 1- and 6-hour-delayed corneal collagen cross-linking (CXL) on wound-healing of experimental alkali burns of the cornea.

Methods: Twenty-four albino rabbits were used. Alkali burns were created using 1 M NaOH. The animals were divided randomly into 2 groups: group 1 (control group, n = 6) and group 2 (experimental group, n = 18). The experimental group was further divided into 3 subgroups as follows: group 2A, untreated (non-CXL) subgroup; group 2B, 1-hour-delayed CXL treatment subgroup; and group 2C, 6-hour-delayed CXL treatment subgroup. All rabbits were examined periodically for 21 days after treatment and then killed. The corneas were excised and histologically examined.

Results: Corneal ulceration, edema, and opacity scores were 4.0 ± 1.64, 1.6 ± 0.65, and 3.5 ± 1.21 in group 2A, 1.5 ± 1.76, 1.3 ± 0.87, and 3.1 ± 1.12 in group 2B, and 2.0 ± 1.90, 1.5 ± 0.79, and 3.3 ± 1.09 in group 2C, respectively. These scores were significantly less in groups 2B and 2C than in group 2A (P = 0.023, P = 0.043, and P = 0.034, respectively). Corneal epithelialization, evident upon staining, was best in group 2B and worst in group 2A (P = 0.012). Histopathology revealed that destruction of corneal collagen fibers and infiltration of inflammatory cells into corneal tissue were reduced in groups 2B and 2C compared with group 2A.

Conclusions: We found that CXL treatment exerted positive effects on severe alkali-induced corneal burns. However, the effects were more pronounced in the 1-hour treatment group. We believe that CXL treatment may be a possible treatment for corneal alkali burn.

Key Words: alkali burn, collagen, cross-linking, matrix metalloproteinase, riboflavin

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Ocular chemical injuries are true ocular emergencies and require immediate and intensive evaluation and treatment. Chemical burns may be caused by either alkaline or acidic agents. Common alkaline agents include ammonium hydroxide, sodium hydroxide (caustic soda), and calcium hydroxide. Alkaline agents are particularly damaging as they exhibit both hydrophilic and lipophilic properties, allowing them to rapidly penetrate cell membranes and enter the anterior chamber. Alkali damage results from the effects of hydroxyl ions causing saponification of cell membranes and cell death, along with disruption of the extracellular matrix.1–3

Corneal collagen cross-linking (CXL) is used clinically to treat keratoconus. As photo-activated riboflavin also exerts antimicrobial effects, various CXL protocols have been used to treat infectious keratitis; such procedures are described by the acronym PACK (photo-activated chromophore for keratitis).4,5 Chromophores typically have a planar ring structure, which readily intercalates between bases of DNA and RNA. Activation triggers oxidation of nucleic acids, causing lesions in chromosomal strands and inhibiting pathogen replication. Release of massive amounts of reactive oxygen species can damage pathogen cell walls, explaining the direct killing effects of CXL on microbes.5

The technique uses riboflavin drops and 365-nm ultraviolet-A light to generate additional cross-links in the cornea. Specifically, riboflavin acts as a chromophore and releases free radicals. In the cornea, free radicals increase the number of covalent bonds among stromal collagen molecules. These additional cross-links enhance the overall biomechanical strength of the cornea.6–11 Moreover, CXL changes molecular structures within the cornea to prevent proteolytic enzymes from binding to specific cleavage sites, thus decreasing the efficacy of collagen-degrading enzymes. Therefore, by increasing biomechanical strength and decreasing proteolysis, CXL has been shown to be an effective treatment for keratoconus and corneal melting in humans.12,13 Only a few studies have evaluated the effect of CXL in terms of prevention of melting in rabbit corneas after alkali burning. However, in these experimental studies, CXL
was administered within the first hour. Given that patients sustaining chemical injuries may be admitted to a health center hours later, it is very important to explore whether delayed CXL is effective. This is the first experimental study to evaluate the efficacy of delayed CXL.

We explored the clinical and histological effects of CXL on corneal melting in rabbits after alkali burns. Our second aim was to determine whether treatment efficacy varied when CXL was performed 1 and 6 hours after burning.

MATERIALS AND METHODS

Animals

The study was conducted between September 2015 and December 2015 at Izmir University and Dokuz Eylül University, Izmir, Turkey. In total, 24 male New Zealand rabbits, weighing 2.4 to 3.2 kg, were used. The study was approved by the Committee on Animal Research of the Dokuz Eylül University Institute for Experimental Medicine Research, Izmir, Turkey. The care, use, and treatment of all animals strictly followed the dictates of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were divided randomly into 2 groups: group 1 (control group, n = 6) and group 2 (experimental group, n = 18). The experimental group (group 2) was subdivided into 3 subgroups: untreated subgroup (non–cross-linked control subgroup) and 1- and 6-hour-delayed treatment subgroups (each n = 6), in which CXL was performed 1 and 6 hours after injury. The experimental subgroups were termed subgroups 2A, 2B, and 2C, respectively.

Alkali Burning

All rabbits in the experimental group were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg) injected intramuscularly into the hind legs, and 0.5% (wt/vol) proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX) was applied topically to induce ocular surface anesthesia. A previously described experimental model of corneal burning was used.14–16 After retracting the lid with a speculum, alkali burns were induced by maintaining round pieces of Whatman filter paper No 50 (diameter: 10 mm), which had been immersed in 0.4 mL 1 M NaOH solution, in contact with the cornea and limbus for 2 minutes. This created severe ocular burns (Roper Hall criteria; grade IV). Each eye was then washed immediately with 10 mL physiological saline solution (0.9% wt/vol NaCl) for 2 minutes.

1- and 6-Hour-Delayed Corneal CXL Treatment

CXL was applied to all animals in groups 2B and 2C. The epithelia were not subjected to the procedure. Riboflavin photosensitizer solution (G. Streuli & Co AG, Uznach, Switzerland) containing 0.1% (wt/vol) riboflavin-5-phosphate and 20% (wt/vol) dextran T-500 was applied at 3-minute intervals for 30 minutes to 9-mm fields (which is a standard method). Corneas were continuously exposed to UV-A irradiation (370 nm; irradiance 3 mW/cm² and surface dose 5.4 J/cm²2; Roithner Lasertechnik, Vienna, Austria) for 30 minutes. The riboflavin solution was simultaneously instilled at 3-minute intervals.

Treatment After Injury

After alkali burning, clinical treatment was initiated in groups 2A, 2B, and 2C by application of 1.0% (wt/vol) prednisolone acetate ophthalmic suspension, preservative-free lubricant eye drops, and 0.3% (wt/vol) ciprofloxacin eye drops. These were all administered 4 times daily for the first 7 days (early phase). After this period, the corticosteroid was stopped and only lubricant eye drops and antibiotic treatments were maintained, for another 14 days.

Clinical Evaluation

All rabbits underwent biomicroscopic examination on days 1, 3, 7, 14, and 21 after alkali burning. Corneal epithelialization, the depth of ulceration, corneal opacity, edema, neovascularization, vessel size, central corneal thickness (CCT), and intraocular pressure (IOP) were clinically evaluated. The percentage of corneal epithelialization was evaluated after staining with fluorescein dye. The areas of regions with epithelial defects (thus absorbing dye) were measured on the photographs. Corneal ulcers were categorized by the lesion depth17: stage 0, no ulcer; stage 1, superficial ulcer; stage 2, medium-depth ulcer; stage 3, deep ulcer; stage 4, descemetocele; and stage 5, corneal perforation. Corneal edema and opacity were graded by 2 masked observers on color microscopic photographs obtained using the method of Yoeruek.18 Corneal opacity was scored on a scale of 0 to 4 (grade 0, completely clear; grade 1, slightly hazy (iris and pupils easily visible); grade 2, slightly opaque (iris and pupils still detectable); grade 3, opaque (pupils barely detectable); and grade 4, completely opaque with no view of the pupils). Edema was scored on a scale of 0 to 2 (0, absence of edema; 1, edema present but mild; and 2, very significant edema). Neovascularization was scored on a scale of 0 to 4 (0, no vessels at the corneal limbus; 1, vessels within 1 mm of the corneal limbus; 2, vessels within 2 mm of the corneal limbus; 3, vessels running from 4 mm over the corneal limbus toward the corneal center; and 4, vessels within 2 mm of the corneal center). The vessel size was scored on a scale of 0 to 3 (0, no vessels; 1, vessels just detectable under the microscope; 2, vessels easily seen under the microscope; and 3, vessels easily seen without the microscope). IOP was measured using a Tonopen XL hand-held device (Medtronic; Jacksonville, FL) and CCT using pachymetry (AccuPachV pachymeter; Acutome, Malvern, PA) before treatment and weekly for 21 days after CXL.

Histological Evaluation

Twenty-one days after ocular burning, all rabbits were killed by intramuscular injection of a 75 mg/mL solution of ketamine hydrochloride (Ketalar; Pfizer, New York, NY) and...
a 5 mg/mL solution of xylazine hydrochloride (Alfaxyn; Ege Vet, Izmir, Turkey). Corneas were processed and analyzed at the Department of Histology, College of Dokuz Eylul University, Turkey. All specimens were maintained in 10% (vol/vol) buffered formalin for 24 hours and then in 70% (vol/vol) alcohol for 3 days. Next, the samples were embedded in paraffin and sagittally cut into 5-μm-thick sections. Hematoxylin–eosin (HE) and Masson trichrome staining were used for histomorphological analysis of all groups. In addition, immunohistochemical (IHC) staining was used to observe the expression levels of corneal collagen type I with the aid of the monoclonal antibody NB600-450 anti-Collagen I (Col-1) (Novus Biologicals, Littleton, CO). Collagen expression levels were scored on a scale of 0 to 4 upon IHC staining [grade 0, absence of staining (no collagen expression); grade 1, weak staining (collagen expressed but weakly); grade 2, moderate staining (moderate collagen expression); grade 3, strong staining (strong collagen expression); and grade 4, very strong staining (very strong collagen expression)]. Histological analysis was performed with the aid of a Nikon Eclipse E100 light microscope (Nikon, Sendai, Japan) under ×40 magnification. Three random fields in the centers of the corneas (adjacent to the lesional areas) and 3 fields in the peripheries (adjacent to the limbi) were analyzed. The epithelia, and the newly formed stroma, were analyzed in fields in the peripheries (adjacent to the limbi) were analyzed. The epithelia, and the newly formed stroma, were analyzed in

Statistical Analyses

Nonparametric Kruskal–Wallis 1-way analysis of variance by rank and the Tukey HSD multiple comparison test were used for the statistical analysis. P < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows software (ver. 11.5; SPSS Inc, Chicago, IL).

RESULTS

Clinical Examination

No cornea became infected and no accidental death occurred. The clinical findings at day 21 for all 24 rabbits are compared (using the nonparametric Kruskal–Wallis 1-way analysis of variance by rank test) in Table 1. The mean corneal ulcer depth was less in group 2B than in group 2A. The mean corneal ulcer depth in groups 2B and 2C differed significantly from that in group 2A. Corneal perforation developed in one rabbit in group 2A on day 20. No corneal perforations were seen in group 2B or 2C. Corneal epithelialization evident upon staining was best in group 2B and worst in group 2A. Corneal opacity and edema scores differed significantly between the treatment subgroups (2B and 2C) and the control subgroup (P = 0.034 and P = 0.043, respectively).

Groups 2B and 2C clearly tended to have lower scores than group 2A. Neither neovascularization nor vessel size scores differed among groups 2A, 2B, and 2C (P = 0.233 and P = 0.131, respectively) (Table 1). Before CXL, the IOP and CCT were similar among the 3 groups (P > 0.05). After CXL, the mean CCT and IOP did not differ significantly among groups (P > 0.05).

Histological Examination (Hematoxylin–Eosin, Masson Trichrome, and IHC Staining)

Group 1

The corneal epithelial layer was normal. Nonkeratinized stratified squamous epithelium was evident. The Bowman membrane under the epithelial layer was homogeneous. Corneal stroma was observed under the Bowman membrane; the sequence and orientation of the collagen fibers were normal. A homogenous corneal stroma was observed under Descemet membrane. The endothelium was a single layer of flat cells. Cell infiltration was not apparent (Figs. 1A, B). The extent of IHC staining was moderate (grade 2, moderate expression of type 1 collagen) (Fig. 1C and Table 2).

Group 2A

Corneal integrity was impaired. The epithelial cells were dysregulated, and the stromal collagen fibers seriously
damaged; the fiber sequence and organization were irregular. Widespread cell infiltration [polymorphonuclear leukocytes (PMNLs) and lymphocytes] was observed. The endothelial layer was impaired (Figs. 1D, E). The extent of IHC staining was strong (grade 3, strong expression of type 1 collagen) (Fig. 1F and Table 2).

**Group 2B**

The corneal epithelial layer was normal. Nonkeratinized stratified squamous epithelium was evident. The Bowman membrane under the epithelial layer was homogeneous. Collagen destruction and sequence impairment were evident in the anterior one third of the corneal stroma. The deep, corneal, stromal collagen fibers were regularly arranged. Leukocyte infiltration was observed in the anterior corneal stroma. Descemet membrane and the endothelium were normal (Figs. 1G, H). The extent of IHC staining was moderate (grade 2, moderate expression of type 1 collagen) (Fig. 1I and Table 2).

**Group 2C**

The corneal epithelial layer was normal. Nonkeratinized stratified squamous epithelium was evident. The stromal layer was damaged, and the collagen fiber sequence was irregular. Especially, the anterior stroma exhibited serious separation of collagen fiber. The anterior and midstroma were infiltrated with PMNLs (Figs. 1J, K). Descemet membrane and endothelium were normal. The extent of IHC staining was very strong (grade 4, very strong expression of type 1 collagen) (Fig. 1L and Table 2).

The mean PMNL infiltration score was 2.5 ± 0.4, 1.0 ± 0.8, and 1.5 ± 0.4 in groups 2A, 2B, and 2C, respectively, and thus did not vary among groups. However, the scores were lower in groups 2B and 2C than in group 2A on day 21. In addition, group 2B exhibited less PMNL infiltration than did group 2C; the difference was significant (P < 0.05). PMNL infiltration and IHC staining scores are shown in Table 2. The extent of separation of stromal fibers and epithelial hyperplasia (indicators of corneal damage) were lower in group 2B than group 2C.

**DISCUSSION**

Alkali burns to the cornea are a major public health problem and can cause corneal perforation and eventually permanent vision impairment. Both experimental and clinical studies have described various treatment modalities.

<table>
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<tr>
<th>PMNL Infiltration Score</th>
<th>IHC Staining Score</th>
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<tr>
<td>Group 2A</td>
<td>2.5 ± 0.4</td>
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<tr>
<td>Group 2B</td>
<td>1.0 ± 0.8</td>
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<tr>
<td>Group 2C</td>
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Values are mean ± SD.
effective in terms of both early- and late-phase wound-healing, which successfully reduced the risk of corneal perforation. However, currently, no optimal treatment has been established.22–27 Recently, riboflavin–ultraviolet-A–induced cross-linking has been used to enhance CXL when treating corneal wounds.

The utility of CXL in terms of corneal healing may be explained by the fact that resistance to collagenase digestion is an essential aspect of cross-linking efficacy during treatment of corneal ulceration.28–32 The stabilizing biochemical effect of cross-linking is explained by changes in the tertiary structure of the collagen fibrils, which prevents access of proteolytic enzymes to specific cleavage sites by virtue of steric hindrance thus created.33 Therefore, after cross-linking, the cornea resists enzymatic digestion for some time. Only a few studies have examined the effects of corneal collagen CXL on wound-healing of experimental alkaline burns.33,34 Gao et al33 found that, in a CXL treatment group, corneal edema, collagen fiber damage, and inflammatory cell infiltration were significantly reduced compared with an untreated group. It was suggested that collagen CXL not only prevented and delayed corneal melting after an alkali burn but also reduced the destruction of corneal collagen fibers and infiltration of inflammatory cells into corneal tissue. Similar findings were reported by Colombo et al,34 who also suggested that CXL might improve the prognosis of acute corneal alkali burns. However, in these studies, the effects of CXL were only evaluated soon after injury. Given that patients with chemical injuries may be admitted to health care centers only hours after injury, early treatment may not be possible. Therefore, it was important to explore whether delayed CXL was effective; we applied treatments 1 and 6 hours after injury. To the best of our knowledge, this is the first study to explore the effect of CXL at both 1 and 6 hours after injury. Unlike 2 previous studies,33,34 we also examined the effects of CXL treatment on CCT and IOP. Our study is the first to describe the effects of CXL treatment on CCT after alkali burning. However, because of our short follow-up time, the utility of measuring CCT in such eyes was very limited.

There were several limitations to our study, including the short observation time and the small number of animals, which limited the statistical power in terms of detection of small differences between the CXL treatment subgroups and the control group. However, it is important to emphasize that the animal numbers were chosen after in-depth discussions with the ethics committee.

In conclusion, we demonstrated that CXL treatment of corneas with alkali-induced injuries decreased corneal inflammation, opacity, edema, and ulcer. Our results also demonstrate that this treatment is safe, and it does not produce complications, such as corneal melting and perforation. Therefore, we believe that CXL treatment, delayed for 1 or 6 hours, may be a therapeutic option for corneal alkali burn. Further studies, including larger numbers of animals observed for longer times, are necessary to verify the effects of corneal collagen CXL after an alkali burn.

REFERENCES


