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Comparative Analysis of Matrix-Regenerating Agent and Corneal Cross-Linking in an Experimental Alkali Burn Rabbit Model

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ABSTRACT

Purpose: This study aimed to investigate the clinical and histopathological effects of corneal cross-linking (CXL) and matrix-regenerating agent (RGTA) treatments after corneal alkali burn.

Materials and Methods: Twenty-four alkali-burned corneas from 24 rabbits were divided into three groups: control, CXL, and RGTA. All animals were investigated for epithelial healing, opacification, ulceration, and neovascularization at days 1, 7, 14, and 21 after the alkali burn. Corneas were excised and sent for histological examination on day 21.

Results: One animal each from the CXL and control groups exhibited moderate ulceration, while no ulceration was observed in the RGTA group. No significant difference was observed among the groups in corneal thickness or corneal opacity measurements at the final visit ($p = .058$ and $p = .544$, respectively). Both RGTA and CXL treatments were effective in terms of epithelial healing and neovascularization ($p = .023$ and $p = .03$, respectively). On histological examination, the CXL and RGTA groups were more effective in treating epithelial loss, stromal edema, corneal vascularization, and leukocytic infiltration than the control group ($p < .05$). The immunohistochemical staining scores of the CXL and RGTA groups for caspase-3, vascular endothelial growth factor, and matrix metalloproteinase-9 in the epithelium and stroma were significantly lower than those in the control group ($p < .05$). In the immunohistochemical examination for inducible nitric oxide synthase, epithelial staining scores were similar among the groups ($p > .05$). In contrast, the stromal staining scores of the CXL and RGTA groups were lower than those of the control group ($p < .05$).

Conclusion: Both CXL and RGTA therapies were effective in reducing anatomical and histopathological complications after corneal alkali burn. Further investigation is needed to determine the optimal timing, duration, and dosage of these treatments.

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Corneal alkali burn; RGTA; regenerating agent; CXL; cross-linking

Introduction

Chemical burns account for 12% of all ocular traumas. Chemical corneal burns can cause serious anterior segment complications secondary to rapid and progressive destruction.[1] The severity of these complications is related to the concentration and pH of the chemical, duration of exposure, and the ability of the chemical to access the ocular surface and tissues.[1,2] The causative alkaline substance should be removed immediately after injury and the pH should be balanced (7.0–7.2). Anti-inflammatory and tissue-supportive therapies should be implemented after the first aid. Surgical procedures such as limbal stem cell transplantation, amniotic membrane transplantation, keratoplasty, and tarsorrhaphy can be considered when other medical treatments are inadequate.

Corneal cross-linking (CXL) is a widely used method that employs riboflavin and ultraviolet-A to strengthen the corneal tissue through formation of intrafibrillar covalent bonds via photosensitized oxidation.[3] CXL has been proven to be an effective and safe method for treating corneal ectasia.[4] Moreover, recent studies have revealed promising results of the CXL procedure in corneal alkali burn.[5,6]

Regenerating agents (RGTAs) act as heparan sulfate analogs in damaged tissues. They bind to extracellular matrix (ECM) proteins and growth factors that protect and reconstruct the ECM by inhibiting various proteolytic enzymes, controlling inflammation, and regulating collagen synthesis.[7] RGTAs have been proven to accelerate corneal healing in many diseases including permanent epithelial defects and neurotrophic ulcers as well as after refractive surgery.[8–10]

In the present study, we aimed to investigate the clinical and histopathological effects of CXL and RGTA treatments after corneal alkali burn. To the best of our knowledge, this study is the first to compare the effects of CXL and RGTA treatments in an experimental corneal alkali burn model.

Materials and methods

Based on sample size calculation using the G*Power software, 24 New Zealand female albino rabbits were used in this experimental study. The animals were randomly divided into three groups: 1) the control group was exposed to alkali burn and underwent no treatment, 2) the CXL group received 30 minutes of CXL at

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24 hours after the injury, and 3) the RGTA group was treated with an RGTA at 24 hours after the injury. The study was conducted according to a protocol approved by the Kocaeli University Animal Experiments Local Ethics Committee (2/5-2017) and followed the Principles of Laboratory Animal Care guidelines. General anesthesia was administered using intraperitoneal injection of 1.1 ml ketamine hydrochloride (0.4 ml Xylazine HCl). Proparacaine hydrochloride was used for topical anesthesia. A filter paper of 8 mm diameter was used to absorb 1 M sodium hydroxide solution for 1 minute and placed onto the center of the right cornea for one minute. The eyes were washed immediately with 500 cc of 0.9% sodium chloride for two minutes.[11] In all the animals, grade IV burns without limbal ischemia were confirmed according to the Roper Hall classification.[12] The left eye was used as healthy control. The burn size was calculated as the average of the horizontal and vertical burn measurements. All animals received 0.1% dexamethasone phosphate four times per day for 10 days and moxifloxacin four times per day for 21 days. Two animals were excluded from the study due to burn sizes greater than 8 mm and two animals from the control group died due to general anesthesia complications.

RGTA and CXL treatment procedures

Animals in the RGTA group received the first drop of RGTA (Cacicol®) at 24 hours after alkali burn. Following the first drop, the treatment was continued with one drop every 48 hours. Other medical treatments were discontinued for a 2-hour washout period.

The CXL procedure (UV-X Illumination System, Peschke Meditrade GmbH, Switzerland) was performed according to the Dresden protocol (3 mw/cm^2 for 30 minutes, 5.4 J/cm^2) at 24 hours after alkali burn.[4] Since the corneal epithelium had peeled up due to alkali burn, no epithelial debridement was performed before the procedure.

Follow-up and examinations

Clinical examinations were performed immediately after alkali burn and were repeated on days 7, 14, and 21. On day 1, anterior segment photography (software version 3.13.1; TOPCON Slit Lamp Microscopy 703963, Tokyo, Japan) and anterior segment optical coherence tomography (OCT) scanning (software version 6.0.11.0; Heidelberg Engineering, Dossenheim, Germany) were performed for all animals after alkali burn injury and before determination of the groups. Central corneal thickness (CCT) was measured manually at each visit using anterior segment OCT. Anterior segment photographs were used to evaluate opacity and vascularization, while fluorescein-stained blue-light photographs were used to monitor epithelial healing (Figures 1 and 2). Vascularization, opacity, and epithelial defects were assessed using Adobe Photoshop® CC 14 (Adobe Inc., San Jose, CA, USA) according to the method described by Saad et al.[13] Epithelial defects, vascularization, and opacities were marked in the photographs and the sizes of the marked areas were obtained in pixels. Subsequently, the entire cornea was marked and converted to measure its size in pixels. The percentages of epithelial defect, corneal vascularization, and opacity were calculated using the following formula: affected area/area of the entire cornea × 100. On day 21, corneal ulceration was examined and classified as follows: 1) no ulceration, 2) superficial ulceration (ulcer limited to the anterior one-third of the cornea), 3) moderate ulceration (ulcer extending to the middle one-third of the cornea), 4) deep ulceration (ulcer extending to the posterior one-third of the cornea), 5) descemetocele, and 6) perforation.[14] Rabbits were killed on day 21, since the maximum inflammation after alkali injury is observed on days 14–21.[15] After the final examination, the corneas were irrigated with 500 cc of 0.9% NaCl to remove agents such as fluorescein. The corneas were excised from the limbus and sent for histological examination.

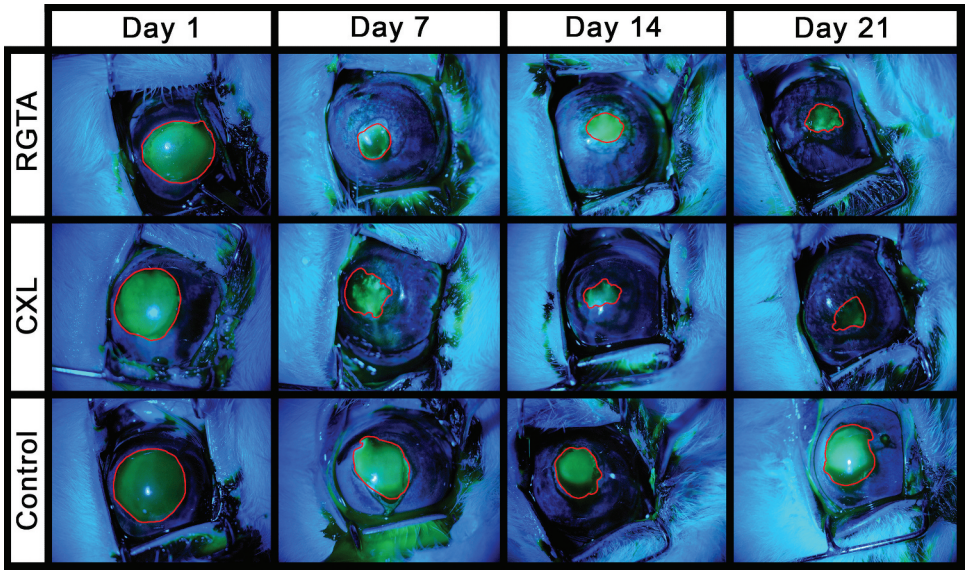


Figure 1. Clinical follow-up of epithelial defects demonstrated by fluorescein-stained blue-light photographs on days 1, 7, 14, and 21 in the RGTA, CXL, and control groups. RGTA: regenerating agents, CXL: corneal cross-linking.

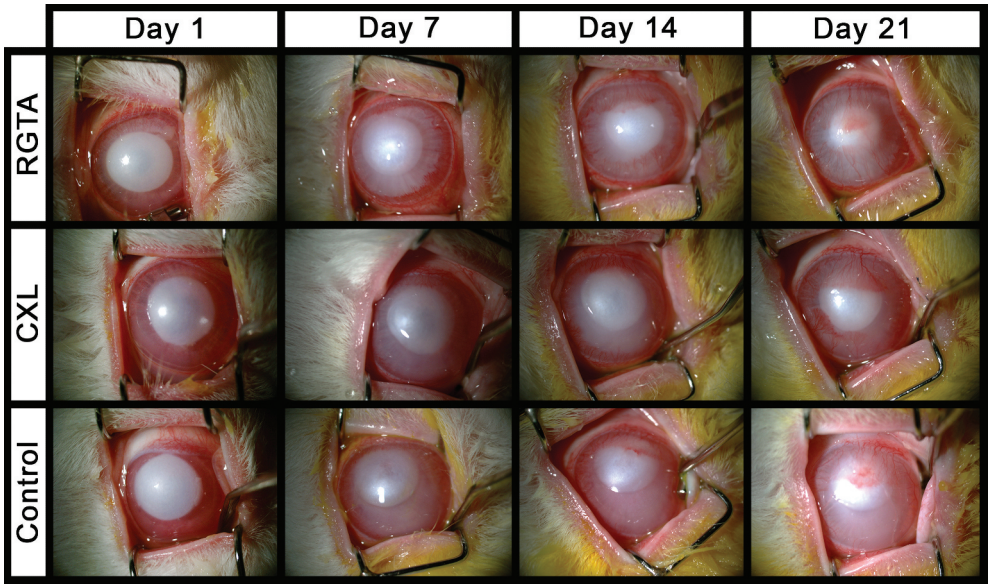


Figure 2. Clinical follow-up of corneal vascularization and opacity using anterior segment photography on days 1, 7, 14, and 21 in the RGTA, CXL, and control groups. RGTA: regenerating agents, CXL: corneal cross-linking.

Histological examination

The corneas were fixed with phosphate-buffered 10% formaldehyde solution, dehydrated in a graded series of ethanol, and embedded in paraffin blocks for histological examination. Serial paraffin sections of 4–5 μm were cut with a microtome (RM 2000; Leica Biosystems Nussloch GmbH, Baden-Württemberg, Germany) and mounted on specially coated slides. The sections were stained with hematoxylin and eosin (Bio-Optica, Milan, Italy) for routine histological examination and Masson's trichrome (Bio-Optica, Milan, Italy) to observe the collagen distribution in the corneal tissue. Histopathological grading of the severity of corneal injury was performed according to method described by Arikan et al.[16] The histopathological grading system included epithelial loss (0 = none, 1 = minimal splitting, 2 = mild erosion, 3 = moderate ulceration, and 4 = strong erosion), corneal hemorrhage (0 = none, 1 = subepithelial, 2 = subepithelial and upper stromal tissue, 3 = subepithelial and partial stromal tissue, and 4 = total stromal tissue), and inflammation (number of polymorphonuclear leukocytes [PMNL] in the amplified area: 0 = none, 1 = 1–5 cells, 2 = 6–15 cells, 3 = 16–25 cells, and 4 = more than 25 cells). Stromal edema classification was modified from a study by Dursun et al. (0 = no edema, 1 = mild edema, 2 = moderate edema, and 3 = severe edema).[17]

All sections were deparaffinized in toluene, passed through decreasing concentrations of ethyl alcohol (100%, 96%, 90%, 70%), and washed for 5 minutes in phosphate-buffered saline. After antigen recovery, primary and secondary antibodies were added to the sections according to the kit protocol. Endogenous peroxidase activity was blocked with 0.1% hydrogen peroxide for 20 minutes. Blocking serum was applied to serial sections for 10 minutes before overnight application of primary antibodies for inducible nitric oxide synthase (iNOS; ThermoFisher, Waltham, MA, USA), vascular endothelial growth factor (VEGF; ThermoFisher, Waltham, MA, USA), matrix metalloproteinase-9 (MMP-9; Abcam, Cambridge, MA, USA), and caspase-3 (dilution rate: 1/100; ThermoFisher, Waltham, MA, USA). All sections were exposed to the secondary antibody for 20 minutes, followed by exposure to streptavidin-peroxidase for 20 minutes. Subsequently, chromogen diaminobenzidine (DAB; Invitrogen, Waltham, MA, USA) was applied. After counterstaining with Mayer's hematoxylin, washing in tap water for 8 minutes, and holding in distilled water for 10 minutes, the slides were mounted with Entellan® (Merck, Darmstadt, Germany).

At least five corneal sections from each rabbit were examined in all the groups. Immunohistochemically positive cells in randomly selected, non-overlapping, full-thickness central corneal columns extending from the anterior surface to the posterior surface of the stroma were evaluated using five microscopic sections from each cornea in all animals. The diameter of each column was 400× microscope field.[18]

Immunostaining for caspase-3, iNOS, VEGF, and MMP-9 in the cornea was evaluated using a previously described technique.[19] The immunostaining scoring system ranging from 0–4 was applied as follows: no (0), very weak (1), moderate (2), strong (3), and very strong (4) expressions in the immunohistochemical examinations. The percentage of positive cells was graded as follows. Grade 0 indicated <5% positive cells, grade 1 indicated 6–15%, grade 2 indicated 16–50%, grade 3 indicated 51–80%, and grade 4 indicated >80% positive cells.[20] All histological examinations were performed by two independent observers blinded to group allocation. Photomicrographs of all sections were acquired with a camera (DMC2900; Leica Biosystems, Baden-Württemberg, Germany) after examination under a light microscope (DM 2000; Leica Biosystems, Baden-Württemberg, Germany).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA). The assumption of normality was assessed using the Kolmogorov-Smirnov test. Normally distributed continuous variables were presented as mean ± standard deviation. Categorical variables were presented as frequencies (percentages). Differences in normally distributed numerical variables among the groups were determined using one-way analysis of variance and Tukey's multiple comparison test. Differences between follow-up time points were analyzed using two-way analysis of variance and Tukey's multiple comparison test. For two-sided hypothesis testing, statistical significance was set at $p < .05$.

Results

Clinical findings

On day 1, no statistically significant differences were observed in injury size ($p = .781$) or corneal thickness ($p = .972$) among the RGTA, CXL, and control groups. The mean CCT of all animals was 652.4 ± 55 µm and the mean burn size was 8.08 ± 0.37 mm.

No statistically significant differences were observed in CCT among the groups on days 1, 7, 14, or 21 ($p < .05$). However, the mean CCT of the control group was higher than that of the other groups and the lowest value of mean CCT was observed in the CXL group on day 21 (Table 1). The mean CCT values were not significantly different between consecutive visits ($p = .17$) in the CXL group, but were significantly different in the RGTA and control groups ($p < .001$).

No ulceration was observed in any of the animals from the RGTA group. In the CXL group, one animal had a descemetocele and one animal had a moderate ulcer. One animal from the control group exhibited moderate ulceration.

Although there was no significant difference among the groups in terms of epithelial defects on day 1 ($p = .702$), epithelial defects were smaller in the CXL and RGTA groups than in the control group in the following examinations ($p = .001$, $p = .008$, and $p = .023$, respectively). Corneal opacity percentages were similar among the groups in all examinations ($p > .05$). Although corneal opacity decreased in all groups from baseline to the final visit, the decrease in opacification was most prominent in the RGTA group. The control group exhibited higher degree of corneal vascularization than the treatment groups at all follow-up time points (Table 2).

Histological findings

Notably, the healthy, CXL, and RGTA groups exhibited similar scores for epithelial loss ($p = .158$). However, epithelial loss was significantly higher in the control group ($p < .001$). The CXL group exhibited the least stromal edema on day 21 (Table 3) and no significant differences were observed between the CXL and RGTA groups ($p = .309$). Stromal edema was considerably higher in the control group than in the other groups ($p < .001$). The corneal vascularization scores of the RGTA and CXL groups were similar ($p = .435$). The control group exhibited significantly higher degree of vascularization than the other groups ($p < .001$ and $p = .002$ compared to RGTA and CXL, respectively). While the RGTA and CXL groups exhibited

Table 1. Mean central corneal thicknesses on days 1, 7, 14, and 21 in the RGTA, CXL, and control groups (µm).

	Day 1	Day 7	Day 14	Day 21	p-value†
RGTA	650.1 ± 29	660.6 ± 112	792.3 ± 238	979.1 ± 252	**p < .001**
CXL	651.2 ± 79	627.8 ± 25	735.4 ± 227	792.1 ± 192	**p = .170**
Control	657.8 ± 59	739.6 ± 83	791.2 ± 139	1126.4 ± 249	**p < .001**
p-value*	**p = .972**	**p = .156**	**p = .856**	**p = .058**	

*Differences among the three groups at each examination.
†Differences among all follow-up examinations within each group.
RGTA: regenerating agents, CXL: corneal cross-linking.

Table 2. Mean area of epithelial defect, corneal opacity, and corneal vascularization relative to total cornea (%).

	Day 1	Day 7	Day 14	Day 21
Epithelial defect				
RGTA	39.2 ± 8.9	11.9 ± 6.3	8.8 ± 4.2	6.7 ± 4.2
CXL	40.7 ± 4.9	9.8 ± 3	9.7 ± 3.2	8.2 ± 4.6
Control	36.99 ± 9.4	22.23 ± 5.5	15.6 ± 2.7	14.93 ± 6
p-value*	0.702	0.001	0.008	0.023
Corneal opacity				
RGTA	38 ± 2.9	35.4 ± 3.7	30.8 ± 5.8	25.2 ± 7.1
CXL	37.2 ± 4	35.3 ± 3.1	33.6 ± 4.6	28.1 ± 3.9
Control	36 ± 5.7	33.9 ± 7	30.2 ± 4.7	28.1 ± 3.4
p-value*	0.740	0.859	0.501	0.544
Corneal vascularization				
RGTA		12.9 ± 4.5	17.9 ± 4.6	33.8 ± 10.6
CXL		9.9 ± 8.1	16.4 ± 7.9	29.1 ± 14.7
Control		19.8 ± 7.2	38.8 ± 23,1	54.3 ± 24.3
p-value*		0.042	0.013	0.03

*Differences among the three groups at each examination.
RGTA: regenerating agents, CXL: corneal cross-linking.

Table 3. Microscopic evaluation of the histological scores for epithelial loss, stromal edema, corneal vascularization, and leukocytic infiltration in the healthy, RGTA, CXL, and control groups on day 21 after alkali burn.

	Epithelial loss	Stromal edema	Corneal vascularization	Leukocytic infiltration
Healthy	0 ± 0	0 ± 0	0 ± 0	0 ± 0
RGTA	0.37 ± 0.83†	0.57 ± 0.9†	0.97 ± 1.02†	0.85 ± 1.05†
CXL	0.08 ± 0.26	0.25 ± 0.43	1.3 ± 1.24	1.05 ± 1.06
Control	3.37 ± 0.83*	1.6 ± 0.23*	2.1 ± 0.87*	2.42 ± 0.98*

†$p > 0.05$ compared to the CXL group.
*$p < 0.05$ compared to the RGTA and CXL groups.
RGTA: regenerating agents, CXL: corneal cross-linking.

similar results in terms of leukocytic infiltration ($p = .777$), severe leukocytic infiltration was observed in the control group ($p < .001$, Figure 3).

The CXL group exhibited the least immunostaining for caspase-3 on day 21 in epithelium and stroma ($p < .001$, Table 4, Figure 4). The RGTA group also exhibited lower immunostaining scores than the control group in epithelium and stroma ($p < .001$). Notably, there was no significant difference between the CXL and healthy groups in caspase-3 immunostaining in epithelium ($p = .804$). Although CXL and RGTA therapies had no significant effect iNOS immunoreactiviy in epithelium ($p > .05$), they significantly reduced iNOS immunoreactivity in stroma ($p < .05$). In the stromal iNOS analysis, the CXL group showed better results in terms of suppressing iNOS immunoreactivity than the RGTA group ($p < .05$). CXL and RGTA treatments significantly suppressed MMP-9 and VEGF expressions in the corneal epithelium and stroma compared to the control group on day 21 after alkali burn ($p < .001$). Although there was no difference in stromal MMP-9 and VEGF expressions between the CXL and RGTA groups ($p = .999$ and $p = .069$, respectively), CXL was more effective than RGTA treatment in suppressing MMP-9 and VEGF expressions in epithelium ($p < .001$ and $p = .035$, respectively).

Discussion

In previous studies, various treatment periods were reported with CXL and RGTA after alkali burns. In two studies involving RGTAs, treatment was started in the first hour after alkali burn.[21,22] In studies involving CXL, treatment was initiated at

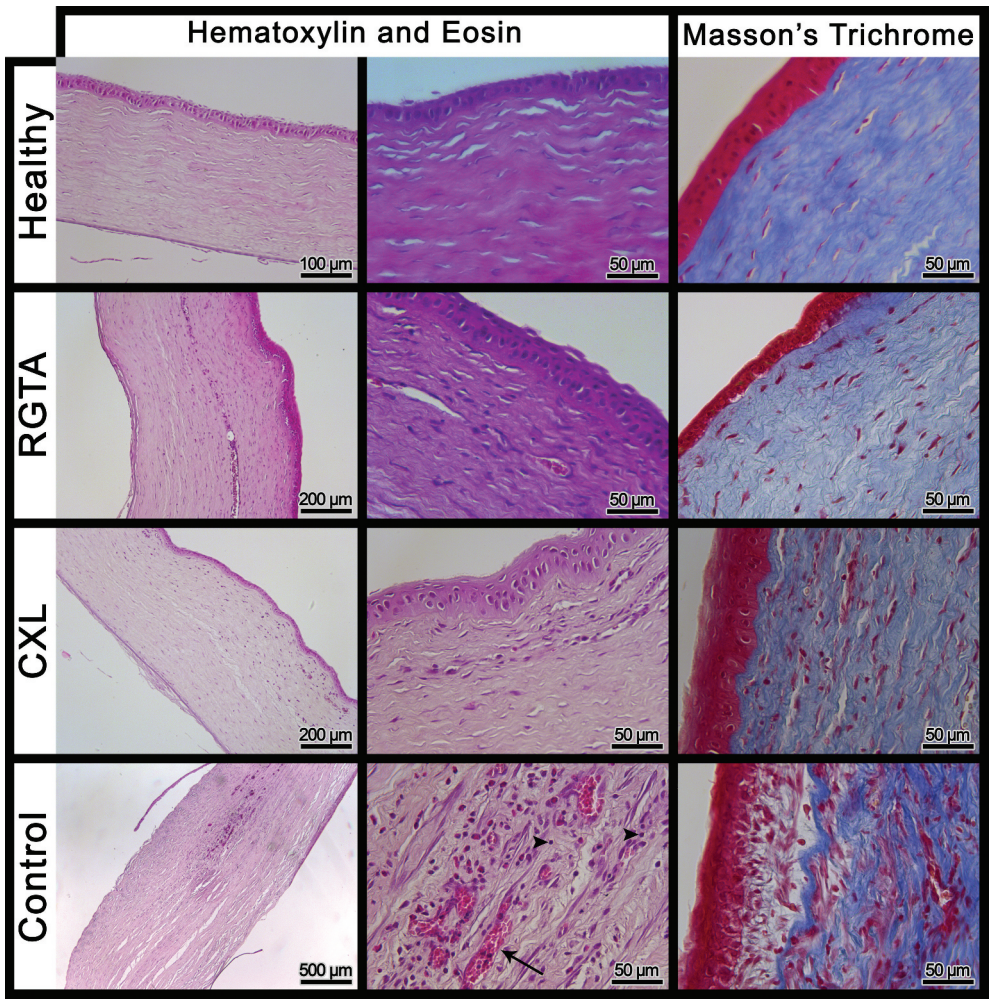


Figure 3. Corneal sections with hematoxylin and eosin and Masson's trichrome (MT) stainings. Compared to the control group, the RGTA and CXL groups exhibited intact epithelium and lower vascularization and lymphocytic infiltration. Collagen sequencing appeared to be more regular in the RGTA and CXL groups than in the control group and it was especially more apparent in the CXL group. Intense vascularization (arrow) and lymphocytic infiltration (arrowheads) were observed in the control group. MT staining showed desquamation of the epithelium and severe stromal vacuolization in the control group. RGTA: regenerating agents, CXL: corneal cross-linking.

Table 4. Microscopic evaluation of caspase-3, iNOS, VEGF, and MMP-9 immunohistochemical scores in corneal epithelium and stroma of the healthy, RGTA, CXL, and control groups on day 21 after alkali burn.

	Caspase-3		iNOS		VEGF		MMP-9	
	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma
Healthy	0.2 ± 0.4	0 ± 0	0 ± 0	0 ± 0	0.12 ± 0.3	0.4 ± 0.2	0.24 ± 0.4	0 ± 0
RGTA	1.66 ± 0.9	1.8 ± 0.7	0.4 ± 0.2†	2.48 ± 0.5	0.88 ± 0.7	1.44 ± 0.9†	1.4 ± 0.9	1.4 ± 0.8†
CXL	0.35 ± 0.4	0.67 ± 0.7	0.2 ± 0.4	0.7 ± 0.6	0.35 ± 0.5	0.97 ± 0.7	0.62 ± 0.6	1.37 ± 1
Control	3.26 ± 0.6*	3.32 ± 0.7*	0.75 ± 0.2	3.2 ± 0.7*	2 ± 1.1*	3.03 ± 0.7*	3.12 ± 0.6*	2.45 ± 1*

†$p > 0.05$ compared to the CXL group.
*$p < 0.05$ compared to the RGTA and CXL groups.
RGTA: regenerating agents, CXL: corneal cross-linking, iNOS: inducible nitric oxide synthase, VEGF: vascular endothelial growth factor, MMP: matrix metalloproteinase.

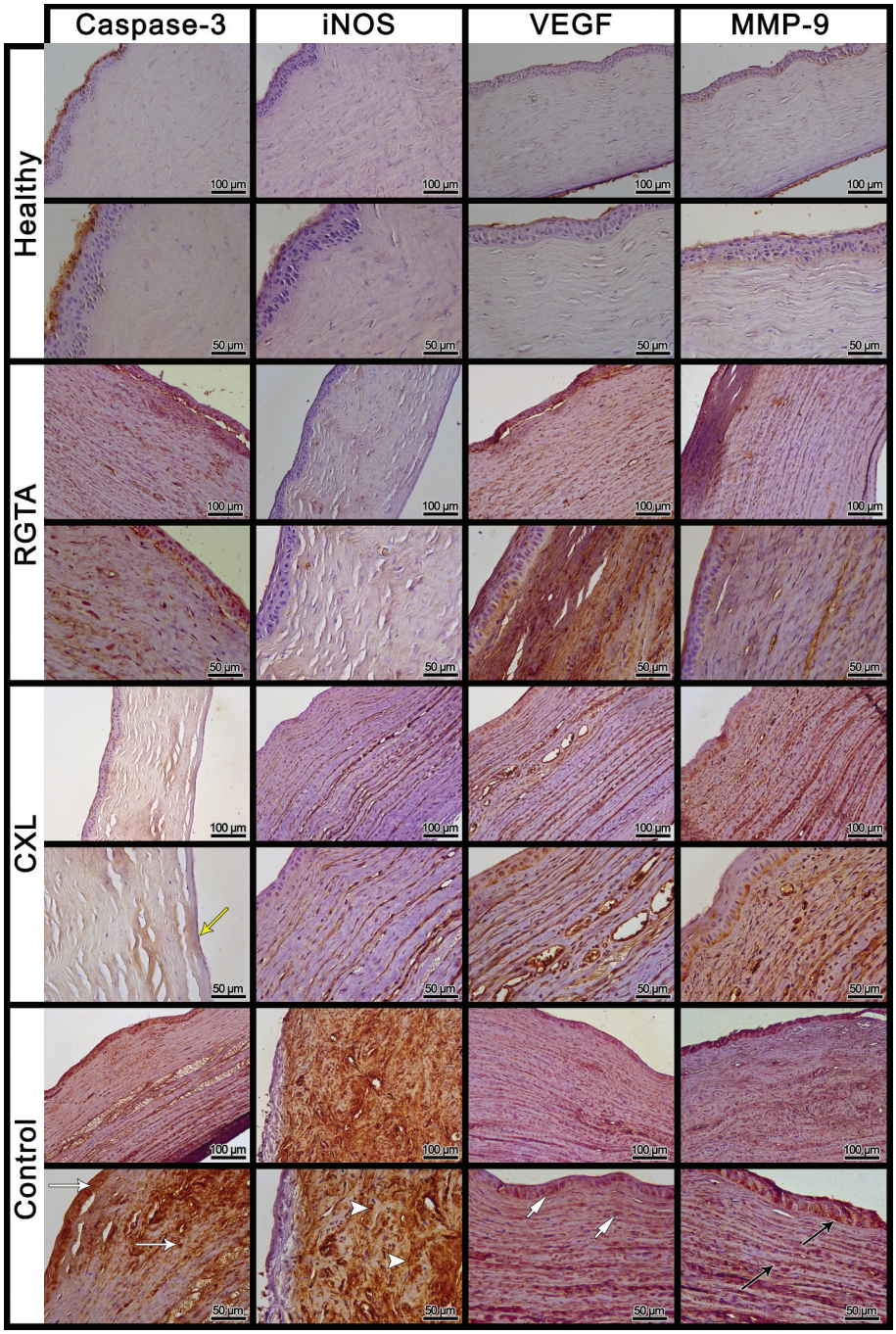


Figure 4. Photomicrographs of immunohistochemical stainings for caspase-3, iNOS, VEGF, and MMP-9. The control group exhibited increased apoptotic cells (white thin arrow) and immunostaining for iNOS (arrowhead), VEGF (half arrow), and MMP-9 (black arrow) compared to the treatment groups. Notably, brown positive cells were uncommon in the endothelium of the CXL group (yellow arrow). RGTA: regenerating agents, CXL: corneal cross-linking, iNOS: inducible nitric oxide synthase, VEGF: vascular endothelial growth factor, MMP: matrix metalloproteinase.

1, 4, 6, 24, and 72 hours.[11,23,24] Although alkali burn should be treated as soon as possible, we initiated the treatments at 24 hours after alkali burn considering real-life situations wherein treatment might be delayed and to add diversity to the literature.

PMNL infiltration, which plays a crucial role in inflammation, is the usual response detected after alkali burn and it causes corneal thinning and ulceration due to collagenase production.[25] Therefore, preventing PMNL infiltration is of great importance in reducing complications. Expression of iNOS is one of the direct effects of an inflammatory response.[26] In corneal inflammatory diseases, high iNOS levels have been observed in vivo.[27] CXL has been proven to directly deactivate the proteolytic enzymes and prevent their release indirectly by damaging leukocytes.[28] RGTA treatment has also been shown to reduce leukocytic infiltration in different tissues.[22,29] Zhu et al. showed that lymphangiogenesis was suppressed on day 7 after CXL in a suture-induced inflammation model in rats, but there was no difference on day 14.[30] Berger et al. reported that there was no change in iNOS expression at 48 hours after CXL in cell culture.[31] On the other hand, RGTA treatment suppressed iNOS release, which was mostly evident in the sixth week.[21] Therefore, post-treatment 48 hours might be too early to examine the anti-inflammatory effect of CXL. The results of the present study suggested that CXL was at least as successful as RGTA treatment in suppressing inflammation at 21 days after the treatment.

MMPs play a vital role in tissue remodeling.[32] MMP-9 is released in low concentration from healthy corneal epithelium and its release increases after ocular surface damage.[14,33] MMP-9 is assumed to induce neovascularization by degrading proteins in the blood vessels' basal lamina and releasing the biologically active form of VEGF,[34] which is required for corneal inflammatory neovascularization[35] Xiaoying Xu et al. showed that MMP-9 expression was higher in the CXL group than in the control group on day 7 after alkali burn. Conversely, MMP-9 expression was lower in the CXL group than in the control group on day 14.[23] Another study showed that epithelial MMP-9 expression was significantly lower in rabbits treated with RGTA on day 4 after alkali burn. Moreover, RGTA therapy suppressed VEGF expression in the third and sixth weeks after alkali burns.[21] Based on previous studies and our results, we propose that early suppression of MMP-9 release by RGTA therapy may be an important factor in preventing corneal ulceration.

CXL and RGTAs have been reported to be effective in the treatment of various corneal ulcers.[8,11,36,37] In our study, CXL was found to be less effective than RGTA treatment in preventing corneal ulceration. Despite promising results in histological examinations, the failure of CXL to prevent corneal ulceration may be due to the late onset of its anti-inflammatory and antioxidative effects. Histological examinations before day 21 might have led to even lower success rates in the CXL group than those observed in the present study.

Subasi et al. showed that corneal alkali burn exhibited remarkably better epithelial healing after CXL than the control and amniotic membrane transplantation groups on days 7, 14, and 18.[11] Colombo-Barboza et al. also reported that the CXL group had a similar extent of epithelial healing compared to the control group on day 7, but exhibited better epithelial healing on days 15 and 30.[24] In contrast to the findings of the present study, the failure of CXL in terms of epithelial healing on day 7 in the aforementioned study can be attributed to the fact that the authors performed the procedure at 1 hour after alkali burn. RGTA treatment has been shown to be an efficient and non-invasive option for corneal epithelial defects including those resistant to conventional therapies.[38,39] In the present study, both CXL and RGTA treatments were effective for epithelial defects in all follow-ups and no treatment exhibited results superior to the other.

Prevention of corneal opacification is a long-term treatment goal after alkali burns. Colombo-Barboza et al. showed that CXL did not differ significantly from the control group in terms of opacification on day 15 after alkali burn. However, opacification significantly decreased in the CXL group when compared with the control group on day 30.[24] Subasi et al. reported no significant differences in corneal opacity between the CXL and control groups on days 7, 14, and 18 after alkali burn.[11] Brignole-Baudouin et al. did not find any significant difference in corneal opacification between the RGTA and saline groups on day 7 after alkali burn.[22] Consistent with other studies, we observed no significant differences in corneal opacity among the CXL, RGTA, and control groups at all follow-up examinations.

Although corneal edema is a rare complication of CXL,[40] it can be used as an alternative treatment option for corneal edema in eligible patients.[41,42] In our study, CCT values showed no significant differences among the groups in all follow-up examinations. In contrast, the RGTA and CXL groups exhibited better outcomes in terms of stromal edema scores than the control group due to higher stromal vacuolization and weak collagen fibers observed in histological sections from the control group. Although CCT values were similar, RGTA and CXL treatments may lead to improvements in visual acuity due to a stronger collagen structure and less vacuolization.

It is unclear whether the results from an experimental rabbit study would be applicable to humans due to the absence of Bowman's layer in the rabbit cornea. Moreover, it should be noted that healthy rabbit corneas are thinner than human corneas. Healthy rabbit CCT, which is approximately 370 μm,[43] is not suitable for CXL.[4] On the other hand, CCTs of the animals increased following alkali burn and reached a safe range to avoid endothelial damage in our study. Although this study is the first to compare the efficacy of RGTA and CXL treatments, we did not include an RGTA/CXL combined treatment group. Moreover, only one protocol was followed at the beginning of the treatment and throughout treatment duration. Ideally, this experiment should be repeated with a larger sample size using different protocols.

In conclusion, both CXL and RGTA therapies were effective and well tolerated after corneal alkali burn. RGTA treatment is associated with accessibility, ease of application, and early onset of antioxidative effect. Larger studies are needed to elucidate the optimal timing, duration, and dosage of these treatments.

Acknowledgments

Clinical investigators:

E. K, MD: Concept, design, data collection or processing, analysis or interpretation, literature search, writing, critical review.

D. P, MD: Concept, design, analysis or interpretation, literature search, writing, critical review.

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Participating investigators:

M. Y. Y, PhD: Concept, design, data collection or processing, analysis or interpretation, writing, critical review.

Y. Y, MD, PhD: Analysis or interpretation, literature search, critical review.

S. F. R, MS: Design, literature search, writing.

Animal research ethics approval

All procedures in this study conformed to the Declaration of Helsinki and Institutional Review Board (Medical Ethical Committee, Kocaeli University Medical Faculty, Kocaeli, Turkey) and "Principles of laboratory animal care" guidelines were followed [Kocaeli University Animal Experiments Local Ethics Committee (2/5-2017)].

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary material.

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