Rose Bengal and Green Light Versus Riboflavin–UVA Cross-Linking: Corneal Wound Repair Response

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Purpose. To study corneal wound healing after two cross-linking techniques using either rose bengal and green light (RGX) or the conventional treatment using riboflavin and UVA radiation (UVX).

Methods. Corneas of New Zealand rabbits were monolaterally treated with UVX (21 eyes) or RGX (25 eyes). Treatments involved corneal de-epithelialization (8-mm diameter), soaking with photosensitizer (0.1% riboflavin in 20% dextran for 30 minutes for UVX; 0.1% rose bengal for 2 minutes for RGX), and light irradiation (370 nm, 3 mW/cm^2, 30 minutes for UVX; 532 nm, 0.25 W/cm^2, 7 minutes for RGX). Contralateral eyes were used as controls. Clinical follow-up included fluorescein staining, haze measurement, and pachymetry. Healing events analyzed after euthanasia at 2, 30, and 60 days included cell death (TUNEL assay), cell proliferation (BrdU [bromodeoxyuridine] immunofluorescence), and differentiation to myofibroblasts (α-SMA [alpha smooth muscle actin] immunohistochemistry).

Results. Re-epithelialization and pachymetries were similar after RGX and UVX. The haze from day 1 to 15 was greater after UVX. Cell death was deeper after UVX, being localized in the anterior and middle stroma, and was superficial (anterior third) after RGX. Cell proliferation appeared after 2 days and was localized in the middle and posterior stroma in the UVX group but was superficial in the RGX group. After 60 days the number of stromal cells had not returned to the control number in either group.

Conclusions. The deeper and longer-lasting cell damage caused by UVX compared to RGX may underlie the slower cell repopulation after UVX and other differences in healing. Shallower damage and a shorter treatment time suggest that RGX may be appropriate for stiffening thin corneas.

Keywords: wound repair, cross-linking, rose bengal, UVA radiation, cornea

Corneal cross-linking is increasingly being used to halt the progression of keratoconus and other ectatic corneal diseases such as post-Lasik ectasia.[1–3] The generally accepted cross-link protocol uses light for irradiation and a photosensitizing dye to initiate photochemical reactions and to shield the corneal endothelium and the retina.[3–9] The photochemical reactions are believed to promote the formation of covalent bonds in and between corneal collagen molecules and with proteoglycans, which result in cornea stiffening.[3] Although different treatment protocols have been proposed, the Food and Drug Administration (FDA)-approved protocol involves the instillation of riboflavin (vitamin B2) as the photosensitizer and ultraviolet light (UVA 370 nm) for irradiation. This is the so-called Dresden protocol and is referred to here as UVX.[10] Emerging techniques include less invasive transepithelial[11] corneal cross-linking following imbibition of riboflavin by iontophoresis,[12] a femtosecond laser-assisted protocol,[13] and use of short, high-power UVA radiation (accelerated cross-linking).[14] An alternative to the UVX treatment has been proposed that uses the dye rose bengal (RB) as a photosensitizer and green light (532 nm) for irradiation and is referred to here as RGX.[15,16]

Both procedures (UVX and RGX) involve photochemical reactions that produce reactive oxygen species and radicals that induce formation of covalent bonds between collagen molecules and between collagen and extracellular matrix molecules.[17] These protein cross-links are believed to stabilize and strengthen the corneal structure, thus increasing corneal stiffness.[10,18–20]

During UVX, riboflavin is applied to the de-epithelialized cornea for 30 minutes, which is then exposed to UVA for up to 30 minutes. Cross-linking with RGX involves applying RB onto de-epithelialized corneas for 2 minutes and then exposure to green light (532 nm) for 7 minutes. Both cross-linking protocols

have been studied clinically and histologically in animal models,[8,15,21,22] but none of these studies directly compared the two methods.

Previous studies have demonstrated that both UVX and RGX increase corneal stiffness, ex vivo and in vivo in rabbit eyes.[15,16,20,23] A significant difference between the two techniques is the region of the stroma in which the cross-links form. Riboflavin diffuses throughout the cornea but after light activation it produces cross-links mainly in the anterior and midstroma regions.[24] In contrast, RB localizes only close to the anterior stromal surface (<120 μm in rabbit cornea) and increases stiffness after irradiation only in the anterior region.[15]

In this study, we sought to directly compare the wound healing processes following UVX and RGX treatments. All procedures were conducted by the same investigators, and the data were processed by the same expert technician.

Materials and Methods

The Animal Ethics Committee at the University of Valladolid approved these animal studies. Animals were cared for following the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female adult albino New Zealand rabbits, weighing 2.5 to 3 kg, were supplied by a center listed in the official register as a provider of laboratory animals (Granja San Francisco, Navarra, Spain).

Treatment Procedure

Forty-six New Zealand white rabbits were divided into two groups. All rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (37.5 mg/kg; Ketolar, Parke Davis SA, Barcelona, Spain) and xylazine hydrochloride (5 mg/kg; Rompun, Bayer, Leverkusen, Germany) in the thigh, followed by topical application of 0.5% tetracaine hydrochloride and 1 mg oxybuprocaine (Colircusi Anestésico Doble, Alconcusí SA, Barcelona, Spain).

One group (21 rabbits) was treated with UVX. The central corneal epithelium was manually scraped with a blunt spatula in the area of an 8-mm-diameter circle that had been demarcated by a trephine. The photosensitizer solution, 0.1% riboflavin (RF) in 20% dextran T500 (Farmacia Magistral, Madrid, Spain) was instilled for 30 minutes. The cornea was then irradiated with UVA light for 30 minutes, using an IROC UVA lamp (370 nm, 3 mW/cm^2; Institute for Refractive and Ophthalmic Surgery, Zurich, Switzerland). Riboflavin was applied every 5 minutes during the light exposure. The limbus was shielded from the UVA radiation by a ∼11-mm inner diameter ring placed immediately in front of the cornea. The contralateral eye was not treated and was used as a control.

The second group (25 rabbits) was treated with RGX. The central 8-mm circle of corneal epithelium was removed as described for UVX. The photosensitizer solution of 0.1% RB in PBS was instilled for 2 minutes. The cornea was irradiated for 200 seconds followed by 30-second restaining with RB, and a second green light irradiation for 200 seconds. The light source was custom-developed and incorporated a 532-nm laser that delivered an irradiance of 0.25 W/cm^2 (MGL-FN-532; Changchun New Industries, Changchun, China) with a collimating lens that provided an 11-mm Gaussian profile beam at the cornea surface. The total fluence was 100 J/cm^2. The cornea was lightly misted periodically with sterile PBS to prevent drying of the surface during light exposure. The limbus was also shielded from the laser beam by a ∼11-mm inner diameter ring.[15,20] The contralateral eye was used as a control.

Clinical Course

The eye anterior segment was evaluated with a surgical microscope (Leica M220 F12; Leica Microsystems, Nussloch, Germany) before and after treatment, at postoperative days 1, 2, 3, 4, 7, 15, and 30 and on day 60 immediately prior to euthanasia. The epithelial wound was stained with sodium fluorescein (Fluotest; Alcon, Cusi, Barcelona) every day until epithelial closure detected by the lack of fluorescein fluorescence. Photographs were taken during the follow-up period and used to measure the area that remained uncovered by epithelium, that is, the fluorescein-positive area. The area function in Cell A software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) was used for this measurement. Corneal pachymetry was carried out with an ultrasonic pachymeter (Corneo-Gage Plus; Sonogage, Inc., Cleveland, OH, USA) at the time points listed above.

Haze was evaluated following Fantes's scale: Grade 0 was a completely clear cornea; grade 1 was more prominent haze not interfering with or affecting refraction; grade 2 was mild corneal haze, obscuring of iris details; grade 3 was moderate corneal haze obscuring of the iris and lens but with the anterior chamber visible; and grade 4 was complete opacification of the stroma in the area of the ablation. Haze grading was performed in a blinded manner.[25]

Tissue Processing and Light Microscopy

Animals were euthanized on days 2, 30, and 60 after treatments by intracardiac injection of sodium pentobarbital (Dolethal 0737-ESP; Vetoquinol, Madrid, Spain) under general anesthesia. Eyes were enucleated and divided into three groups: group 1, UVX; group 2, RGX; and group 3, contralateral untreated eyes (control). Corneas were then fixed in 4% buffered paraformaldehyde and embedded in paraffin. Five-μm-thick sections were stained with hematoxylin-eosin (H-E). Sections were examined under an Olympus BX41 microscope (Olympus Life Science, Hamburg, Germany), and photomicrographs were obtained with an Olympus DP20 Digital Camera. Quantitative measurements of the photographs were made using the program Cell A (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Cell Counting

Corneal thickness and cell counting were carried out using methods described in a previous study.[22] Briefly, in each cornea, three measurements of corneal full thickness were taken at ×40 magnification. Cells were then counted at ×100 magnification using the Touch Count function from Cell A software (Olympus). All cells below the epithelium to above the endothelium in columns of 90,000-μm^2 area were counted in the center of the cornea and limbus. Each column was divided into anterior, medial, and posterior layers, each approximately 30,000 μm^2. All H-E–stained sections were prepared identically to facilitate comparison.

Cell Death: TUNEL Assay

In order to detect DNA fragmentation associated with apoptosis, terminal uridine nick-end labeling (TUNEL) assays were performed in deparaffinized sections following the manufacturer's instructions (TUNEL, G3250; Promega Corp., Madison, WI, USA).

Cell Proliferation

One hour before euthanasia, the animals received an intramuscular injection of 5-bromo-2′-deoxyuridine (BrdU, 10 mg/mL, 5

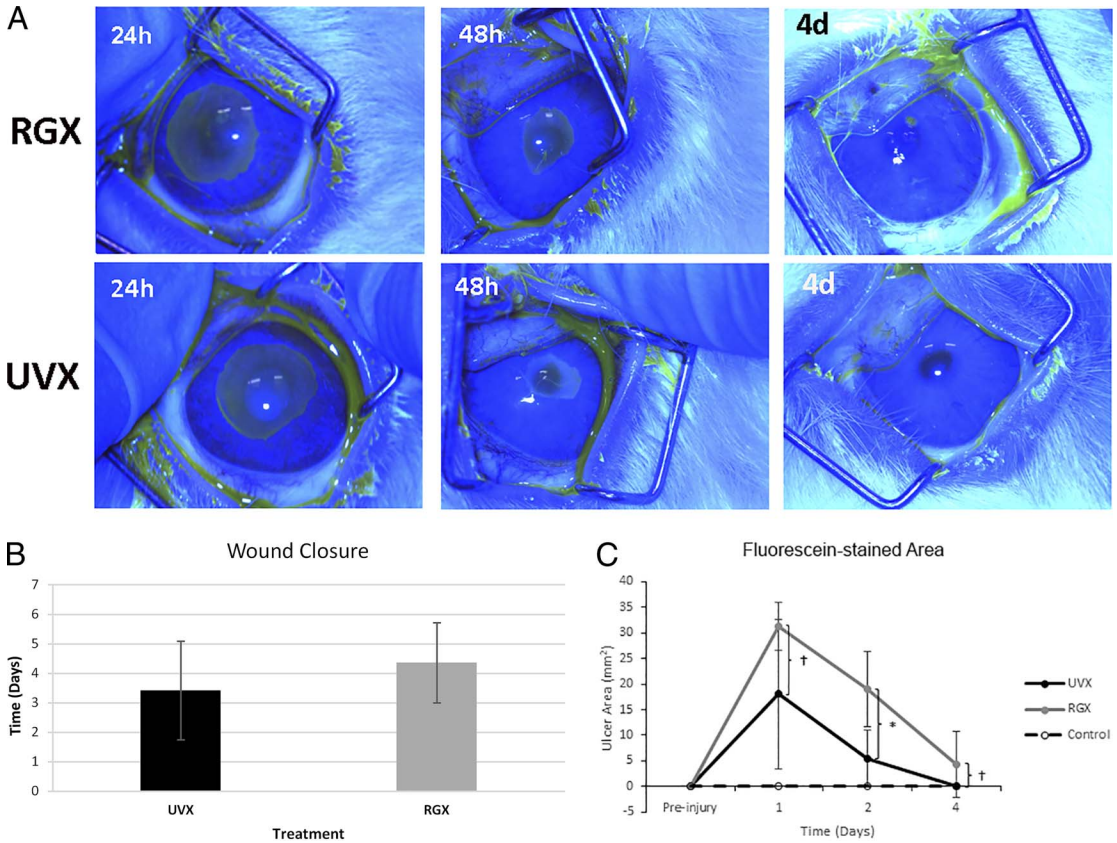

Figure 1. Re-epithelialization after UVX and RGX treatments as assessed by fluorescence of fluorescein from nonepithelialized areas. (**A**) At day 4, fluorescence was detectable in some RGX-treated corneas whereas in the UVX group the epithelium was totally closed. (**B**) Mean time in days for complete re-epithelialization of the treated cornea areas. (**C**) Fluorescein-stained area measured on photographs of corneas at 1, 2, and 4 days post treatment. $^{*}P \leq 0.001$; $\dagger P \leq 0.05$.

mL/kg; Sigma-Aldrich Corp., St. Louis, MO, USA), a DNA synthesis marker. Sections were deparaffinized and treated with 2 N HCl at 37°C for 1 hour, then incubated with mouse monoclonal IgG anti-bromodeoxyuridine (anti-BrdU) (Agilent Technologies, Santa Clara, CA, USA) for 30 minutes at room temperature. The secondary antibody was fluorescein goat anti-mouse IgG (1:100; Molecular Probes, Leiden, The Netherlands) in Tris-buffered saline. Control sections were prepared by omission of the primary antibody.

Myofibroblastic Differentiation

Myofibroblasts were identified by staining with anti-alpha smooth muscle actin (α-SMA) monoclonal antibody (mouse clone 1A4; Dako, Glostrup, Demark). The secondary antibody was Texas red goat anti-mouse IgG (Molecular Probes). Nuclei were stained with DAPI (Molecular Probes). Limbal blood vessels were used as positive controls, and omission of the primary antibody provided negative controls. Immunofluorescence sections (from TUNEL, BrdU, and α-SMA) were examined under an Axiophot fluorescence-incorporated microscope (Zeiss Axiophot HB0-50; Carl Zeiss, Oberkochen, Germany) and photomicrographs were captured using the AxioCam HRc Digital Camera and Axiovision release 4.8 software (Carl Zeiss).

Statistical Analysis

Quantitative characteristics were expressed as mean ± standard deviation (SD). Normality assumptions were checked by the Shapiro-Wilk test. Differences between the means of two independent groups were tested by Student's *t*-test or the nonparametric alternative, Mann-Whitney *U* test, if the normality assumption was not valid. Levene's test was used to check homogeneity of variance. When this assumption was violated, the Welch test was used. A 1-way analysis of variance (ANOVA) was utilized to determine whether there were any statistically significant differences between the means of two experimental groups and the control group. Mean differences over time were tested by an ANOVA with repeated measures. The sphericity assumption was checked by Mauchly's test and, in a case of violation of sphericity, the Greenhouse-Geisser correction was used. Bonferroni post hoc testing was used to determine where differences lay in a pairwise analysis. When data had marked deviations from the normality assumption, Kruskall-Wallis test was used followed by the post hoc analysis based on Mann-Whitney *U* test. Statistical analyses were performed using the R Project for Statistical Computing R version 3.4.2 (http://www.R-project.org; in the public domain). $P < 0.05$ was considered to be statistically significant.

Results

Clinical Assessment

Fluorescein testing was positive from day 1 after treatments until day 3.66 ± 2.1 in the UVX group and 4.3 ± 1.36 in the RGX group (Fig. 1B). The fluorescein-stained area, measured on photos of the corneas, was significantly larger in the RGX group at days 1, 2, and 4. In the RGX group the fluorescent area decreased significantly with time whereas in the UVX

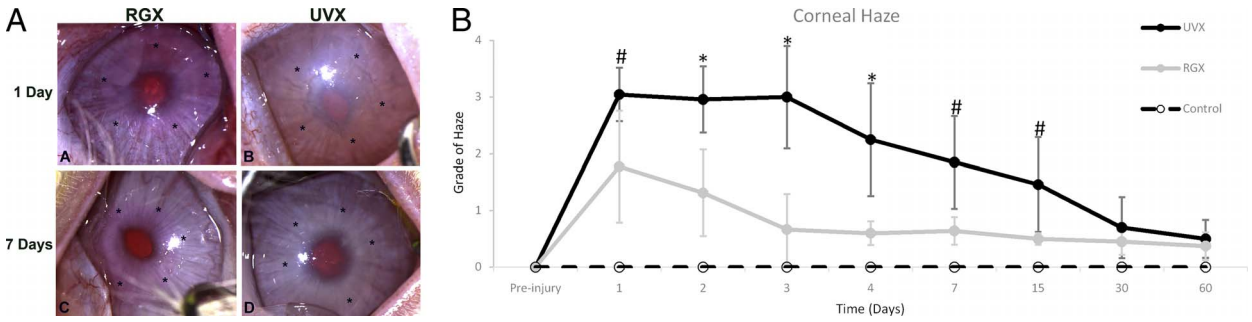

Figure 2. Corneal haze after UVX and RGX treatments. (**A**) Photographs of corneas at days 1 and 7 post treatment. *Asterisk*: area with haze. (**B**) Haze was evaluated from 1 to 60 days using the scale developed by Fantes et al.[25] Differences between groups were statistically different from day 1 to 15. $^{*}P \leq 0.001$, $^{\#}P \leq 0.01$.

group, the areas on day 1 and 2 were significantly different from that for day 4.

Corneal haze in corneas of both groups is shown in Figure 2A. At day 1, differences between UVX and RGX were clear. In UVX corneas a subepithelial haze was visible (grade 3: opacity easily visible on direct focal illumination), whereas in RGX corneas the haze grade was lower and a pink color in the surface of the cornea was apparent. At day 7, haze decreased in UVX corneas (grade 2: mild corneal haze, obscuring of iris details) and RGX corneas still showed pink color, which remained until the end of the study. Following the scale for corneal haze developed by Fantes et al.,[25] the haze severity decreased from grade 3.04 ± 0.47 in the UVX group and 1.77 ± 0.98 in the RGX group at 1 day to 0.50 ± 0.33 and 0.37 ± 0.24, respectively, at day 60. Differences between UVX and RGX were statistically significant from day 1 to 15, the haze being greater in the UVX group (Fig. 2B). The differences between both groups and control were not statistically significant at day 30 nor at day 60.

Corneal thickness measured by pachymetry reached a maximum at day 2 (Fig. 3). At this time, corneal thickness in both treated groups was statistically significantly different from that of the control corneas (RGX $P \leq 0.001$ and UVX $P \leq 0.01$). Corneal thickness then decreased until day 4, reaching values similar to that of the controls. No significant difference was found between groups and control between 4 and 60 days (Fig. 3).

Corneal Morphology

Hematoxylin–Eosin Sections. At day 2, the epithelium did not totally cover the stroma and the uncovered area was larger for RGX than for UVX (Figs. 4A, 4B). After both cross-linking treatments, the stroma showed an area depleted of keratocytes, and this area was larger after UVX than after RGX treatment. Sections from UVX-treated corneas at 2 days displayed cells in the deepest one-third of the stroma. Sections from RGX group corneas at 2 days showed cells in two-thirds of the stroma. In both cases the superficial cells displayed picnotic nuclei (darkly stained cells) that correspond with dying cells (Figs. 4A, 4B). At day 30, the epithelial thickness had increased in both treated groups, reaching approximately the epithelial thickness of the control group, and the stroma seemed to be repopulated with cells (Figs. 4D, 4E). At day 60, after either treatment, it was possible to observe similar epithelium in both groups and a stromal strip devoid of keratocytes near the anterior surface (Figs. 4G, 4H). As is shown at greater magnification in the detailed photographs in Figures 4G and 4H, the thickness of this cell-free strip was greater after UVX than after RGX treatment (84.0 ± 41.9 and 41.7 ± 18.2 μm, respectively), and this difference was statistically significant ($P = 0.0266$).

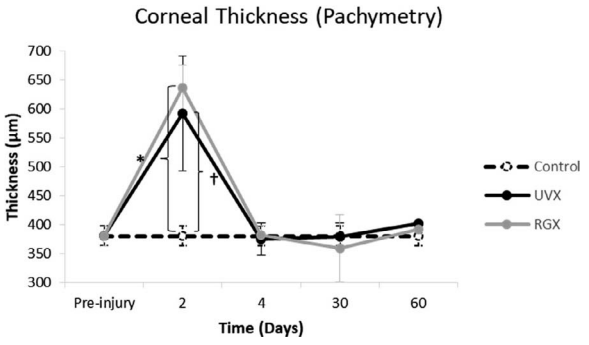

Figure 3. Cornea thickness after UVX and RGX treatments. Measurements were made by pachymetry from preinjury to 60 days post treatment. $^{*}P \leq 0.001$; $\dagger P \leq 0.05$.

Corneal Thickness. Sections from both RGX and UVX group corneas showed substantial edema at day 2 (Figs. 4, 5), which was greater after UVX treatment (Figs. 4B, 5). The thickness then decreased and by day 30 reached the control value (Figs. 4D, 4E, 5). After 60 days, the thickness in the UVX group increased and the difference between the treatment groups was significant, although each treatment group was not different from the control group (Figs. 4, 5).

Epithelial Thickness. At day 2, re-epithelization was more complete in the UVX group than in the RGX group ($P \leq 0.01$), although in both groups the epithelial thickness was significantly different from the control (Fig. 6). Re-epithelialization of the entire central cornea had not occurred in the RGX group at day 2, as seen in Figure 1, and the thickness values were significantly different from control ($P \leq 0.001$) and also from the UVX group ($P \leq 0.001$) (Figs. 4, 6). At days 30 and 60 there were no significant differences between both groups and control or between treatment groups.

Cell Density in the Stroma. At day 2, the number of stromal cells in the wound area was significantly lower after both treatments ($P \leq 0.001$ compared to control), although there was not a significant difference between treated groups (Fig. 7A). At day 30, these differences from control were maintained, although the number of cells increased substantially ($P \leq 0.01$). At day 60, cell density in the UVX group decreased, making the differences significant compared to the control ($P \leq 0.001$). In the RGX group the cell density was higher than in UVX corneas, although it was still significantly lower than the control ($\#P \leq 0.01$) (Figs. 4, 7A). In the peripheral area, the number of cells was not affected by the treatments. Only at day 60 the RGX group showed a significant decrease ($\#P \leq 0.01$) with respect to the control (Fig. 7B).

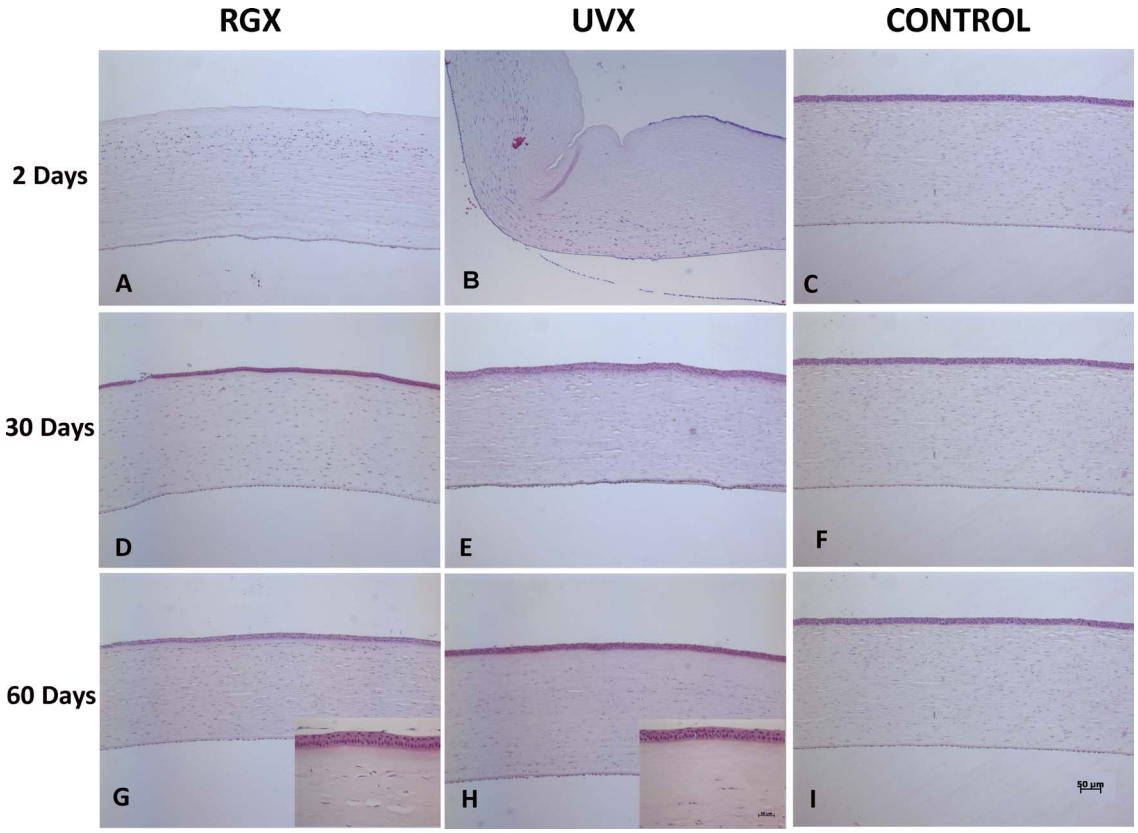

FIGURE 4. Hematoxylin–eosin-stained sections from RGX (**A**–**G**)- and UVX (**B**–**H**)-treated corneas and from untreated control corneas (**C**–**I**). (**A**, **B**) Two days after treatments, the corneas show a depletion of cells in the anterior third of the stroma (RGX) and anterior and middle stroma (UVX). Darkly stained cells correspond to dying cells with picnotic nuclei. (**D**, **E**) Thirty days post treatment, stromal repopulation with cells is apparent. (**G**, **H**) Sixty days after treatment, corneas show a strip below the epithelium that is depleted of keratocytes and that is wider in the UVX group. Magnification ×50; *scale bar*: 50 µm. In (**G**, **H**), *insets* show in greater detail the thickness of the cell-depleted strip. Magnification ×400; *scale bar*: 50 µm.

Given that in cross-linking treatments the depth of the damage is an important landmark, we evaluated the density of cells in the anterior, middle, and posterior stroma (Fig. 8). We found that both treatments produced remarkable damage in the anterior zone that was visible on days 2, 30, and 60 as a significant decrease in the number of cells compared to the control ($^*P \leq 0.001$) (Fig. 8A).

In the middle stroma at day 2, the cell density was significantly lower in the UVX group compared to the control group, but there was no significant difference between RGX-

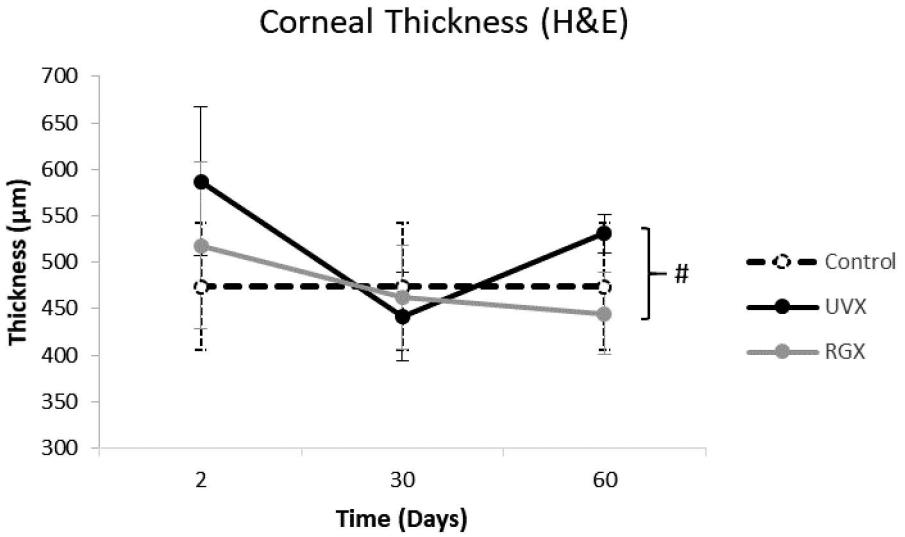

FIGURE 5. Corneal thickness measurements on sections obtained 2, 30, and 60 days post treatment with RGX and UVX. Sections were stained with H-E. $\#P \leq 0.01$.

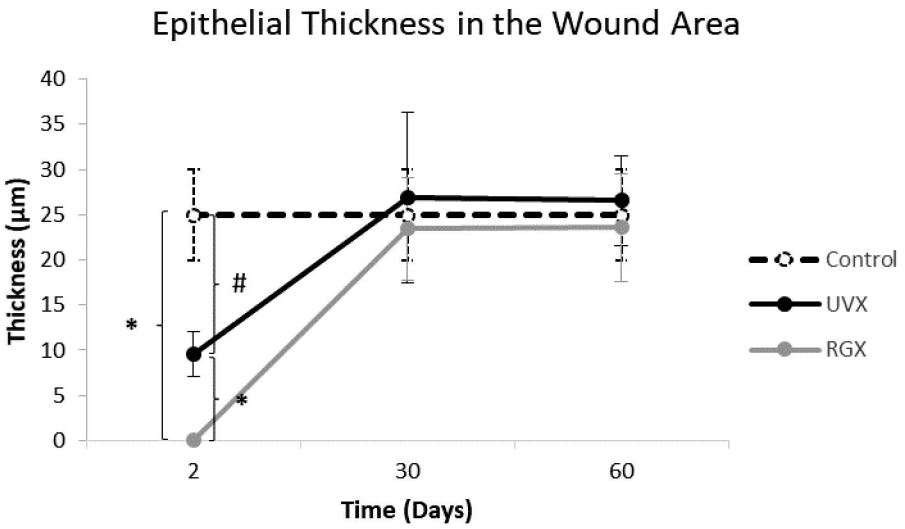

FIGURE 6. Time course for epithelial thickness evolution after RGX and UVX treatments. *$P \leq 0.001$; #$P \leq 0.01$.

treated corneas and controls. At day 30, the cell density greatly increased in the UVX group but not in the RGX group. At day 60, unexpected decrease in cell density was observed in the UVX group, producing a statistically significant reduction from the control group ($P \leq 0.01$). The RGX group maintained approximately the same number of cells (Fig. 8B).

In the posterior stroma on days 2 and 30, no differences in cell density were observed between either treatment and control. However, after 60 days, there was a nearly significant difference ($P = 0.054$) between the corneas treated with UVX and the controls (Fig. 8C).

Cell Death

Sections labeled using the TUNEL assay were evaluated 2, 30, and 60 days after treatment. At day 2, positive cells were observed in the anterior stroma of the RGX corneas in an area coinciding with the scraped area. In contrast, sections from the UVX group at 2 days showed a cell-depleted zone in the anterior stroma and numerous positive TUNEL cells in the middle and, in some cases, in the posterior stroma (Figs. 9A, 9B). At day 30, the UVX group showed scattered labeled cells in the stroma especially in the anterior zone. There were no positive cells in the RGX group. At day 60, numerous TUNEL-positive cells were found in the anterior third of the stroma in the UVX group (Fig. 9C) but not in the RGX group.

Cell Proliferation

At day 2, after either treatment, numerous BrdU-positive cells were observed at the limbus, indicating that this zone was preserved (Figs. 10C, 10F). A line of BrdU-positive cells was observed throughout the epithelium after both treatments (Fig. 10). The stromal cell proliferation was very different. In sections from the UVX group, BrdU-positive cells were found in middle and posterior stroma, even close to Descemet's membrane (Figs. 10D, 10E), whereas in sections from the RGX group these cells were located only just below the epithelium (Figs. 10A, 10B). At day 30, no BrdU-positive cells were observed in the stroma from the UVX or RGX groups.

Myofibroblast Differentiation

No α-SMA-positive cells were observed at either 30 or 60 days in the RGX and UVX groups. The artery walls in the limbus were used to validate the effectiveness of the antibody used.

DISCUSSION

The current study examined the main healing processes after two different corneal cross-linking treatments and revealed some interesting differences as well as some similarities

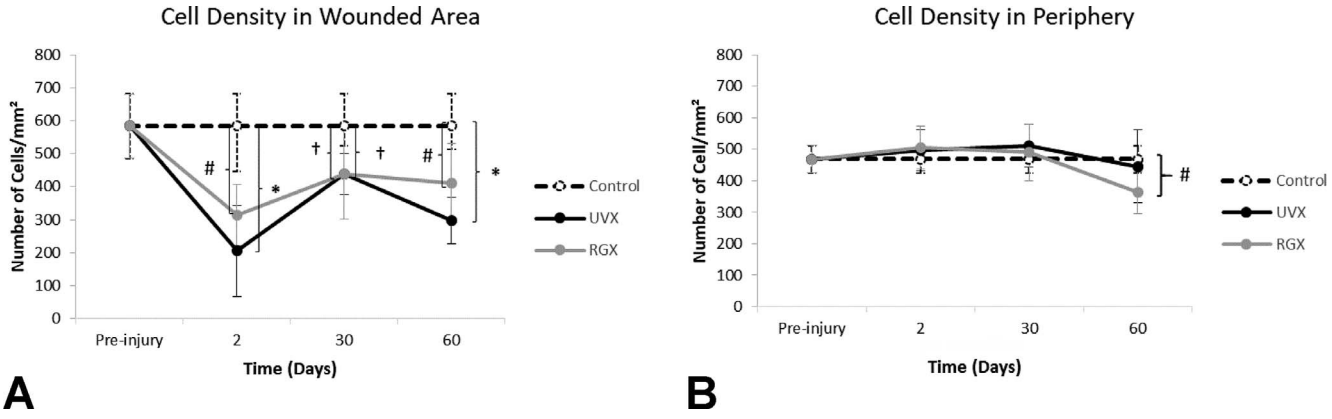

FIGURE 7. Number of stromal cells after treatment with UVX or RGX. Measurements were made pretreatment and at 2, 30, and 60 days post treatment. (**A**) Cell number in the center of cornea. (**B**) Cell number in the limbal zone. *$P \leq 0.001$; #$P \leq 0.01$; †$P \leq 0.05$.

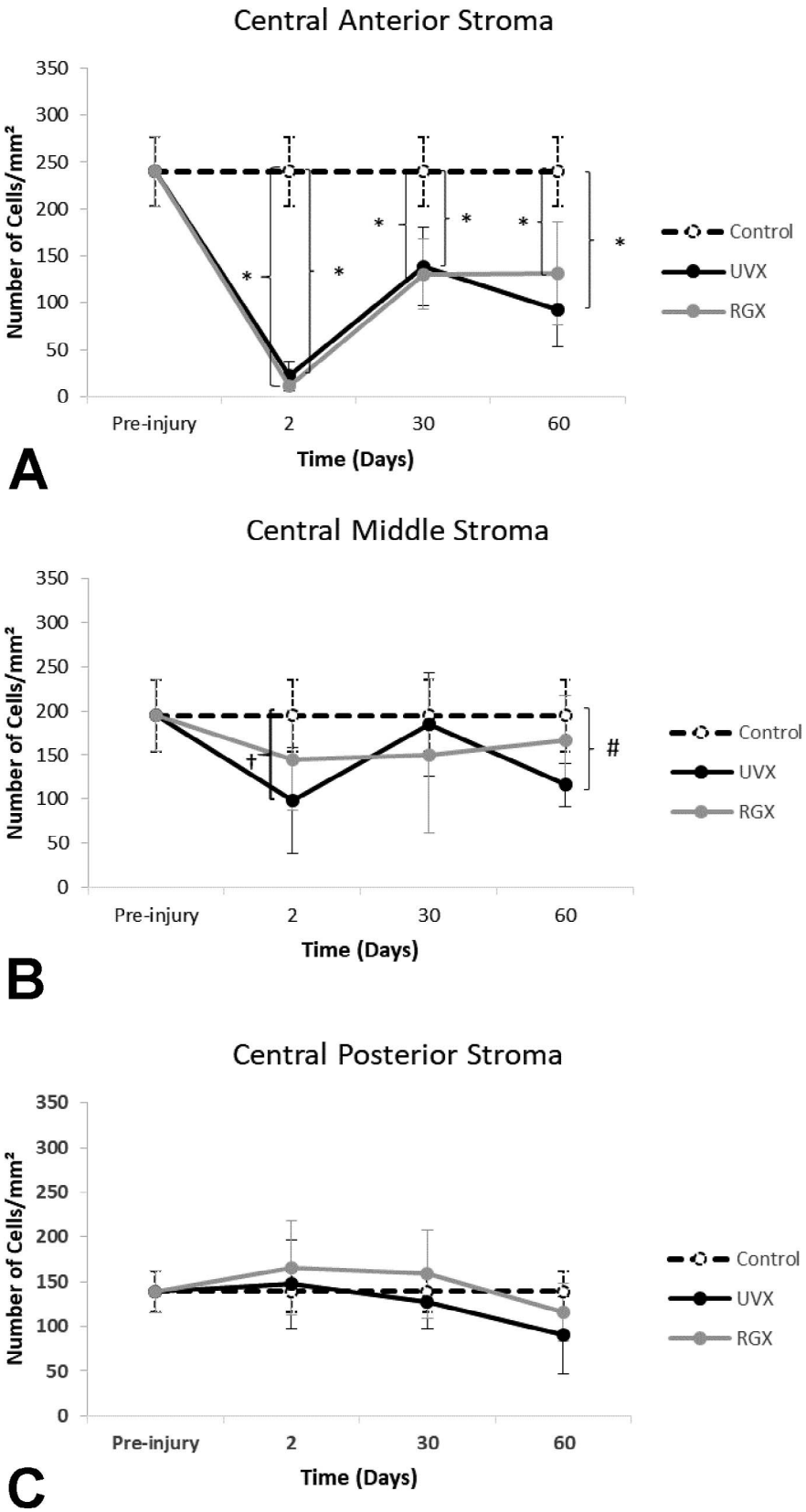

FIGURE 8. Number of cells in the treated zone at varying depths in the stroma. (A) Anterior third of the stroma, (B) middle third, and (C) posterior third. *$P \leq 0.001$; #$P \leq 0.01$; †$P \leq 0.05$.

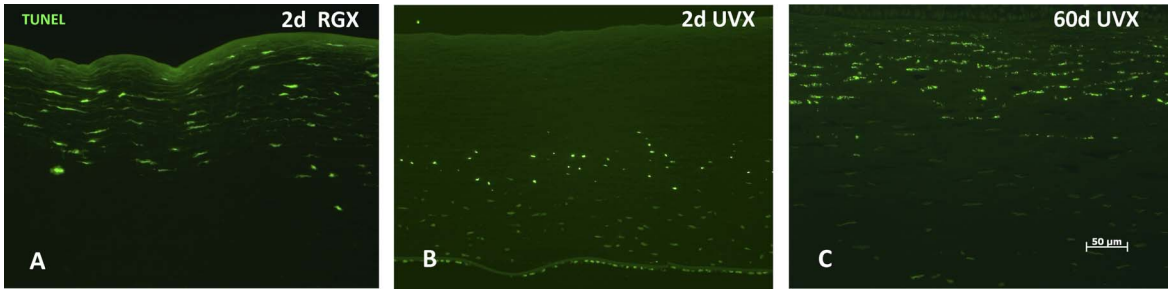

FIGURE 9. Cells undergoing apoptotic death in corneas 2 days after treatment with RGX (**A**) and UVX (**B**). Cornea 2 months after UVX treatment (**C**).

between the responses. Several of the major differences appeared to be related to the greater stromal depth at which UVX treatment causes damage compared to the RGX treatment. These differences included greater depth of stromal cell depletion after UVX and thus a longer time course of regeneration, and development of stromal haze in UVX-treated corneas. UVX and RGX treatments elicited similar re-epithelialization kinetics, stromal cell repopulation kinetics, and an anterior stromal strip devoid of cells that was still observed after 60 days. A novel finding was a second wave of stromal cell death observed at 60 days that appeared only after UVX treatment.

Because RGX has been proposed as a new approach to cornea cross-linking that produces cross-links only close to the anterior stroma surface, a direct comparison with UVX of the corneal repair processes was needed for better evaluation. We had previously reported that the healing processes after RGX treatment were very similar to those observed in corneas that had only been de-epithelialized.[22] Although several experimental studies of wound healing after UVX have been reported, direct comparisons with the responses to RGX described in our previous report were problematic because of variations in the pretreatment protocols and measurements across previous published studies. For example, RF was not applied prior to irradiation in one study[8] whereas in others a presoaking time of 5 to 10 minutes was used.[7,9,21,26] Also the time point for detection of cell death[8,9,21,26] and repopulation of the stroma[7,9,21,26] varies between studies and varies from that used in our previous study of RGX.[22]

To provide a thorough comparison of the cornea healing response from both clinical and histologic measurements, the same investigators performed and analyzed the data from both treatments (RGX and UVX), with the same breed of rabbits (New Zealand white) kept under the same housing conditions and being subjected to the treatments in the same sessions. Overall, the wound healing stages were similar to those after other corneal refractive surgery techniques described in previous work.[9,27,28]

The clinical signs in both treatment groups were similar regarding epithelial wound closure and corneal thickness, primarily driven by the responses due to de-epithelialization alone.[22] The statistically higher central corneal haze grade in the UVX group after 7 days post treatment could be related to the greater depth of damage in the UVX treatment. In addition, a light pink color was visible in the RGX group from 1 day to the end of study. Since this did not correlate with the grade of haze, it may not be detrimental to the visual quality of these corneas. The slight pink staining of the cornea suggests that a small amount of RB remains staining the collagen fibers.

From the histologic assessment, the most noticeable difference between groups was the cell death location, which in the UVX group was found in the anterior and middle zones whereas in the RGX group it was localized only in the anterior zone and was similar to that reported for de-epithelialization

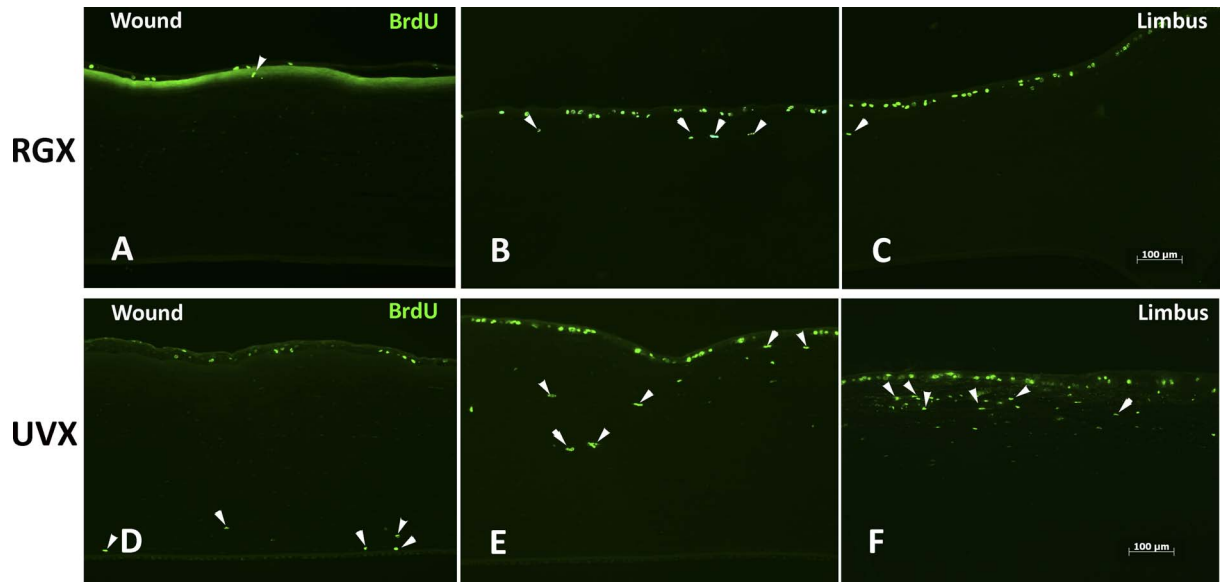

FIGURE 10. Cell proliferation detected by BrdU labeling 2 days after RGX (**A–C**) or UVX (**D–F**) treatments. Three areas are shown: treated area (**A–D**), intermediate zone between the treated area and the limbus (**B–E**), and limbus (**C–F**). *Arrowheads* point to labeled cells assessed by BrdU assay.

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alone.[22] Our results for UVX treatment are consistent with previous studies in rabbits in which massive cell death was reported throughout the stroma 3 days after treatment,[9] or to nearly the full stromal thickness 24 hours after treatment.[7] Two days after both treatments, proliferation appeared in limbal stem cells and in the epithelium, indicating that the stem cells were not damaged.

Surprisingly, dead cells appeared in a second wave 2 months after UVX treatment. This was very clear in our cell-counting results since the number of cells declined at 60 days in the anterior and middle stroma (Fig. 8), and results of the TUNEL assay showed a substantial number of labeled cells in the anterior stroma (Fig. 9C). This event had not been described in other experimental studies that reached this time point where "only rare TUNEL positive cells were noted" (p. 335).[7] This second wave of cell death could have been caused by UVA-induced DNA damage in cells in the periphery, close to the wound or in the deeper stroma. If these cells are not able to repair their DNA, after several mitoses they may suffer later apoptosis.[29,30] This hypothesis is supported by observations by Mastropasqua et al.[31,32] on in vivo corneal confocal microscopy images from human patients that show a reduction of cellular density 1 year after UVX treatment.

In the UVX group, cell repopulation by cell division started deep in the stroma, whereas it was superficial in the RGX group (Fig. 10), again reflecting shallower light-initiated damage in the RGX compared to the UVX group. Several previous studies reported on repopulation of the stroma post UVX treatment, although none of them labeled the proliferating cells or counted the number of cells as was done in our study. However, the results of those studies are consistent with our findings. For example, using linear backscattering contrast in reflective fs-CLSM, a weak autofluorescent signal associated with keratocytes was observed 6 days post treatment.[26] Also, in studies evaluating H–E sections, repopulation had been observed at 7 days in the posterior corneal stroma,[9] and at 6 weeks, complete keratocyte repopulation had been described by Wollensak et al.,[9] although Kruger et al.[26] observed a lower density of cells. In addition, in the report by Armstrong et al.[7] the restoration of keratocyte density appeared complete at 2 months.

In our study, the regeneration did not reach the number of the cells in controls (Fig. 8), and in fact, an anterior cell-free strip in the stroma was visible at the end of the study (60 days), with this cell-deployed layer appearing wider in the UVX than in the RGX group (Figs. 4G, 4H details). We had previously postulated that this cell-free zone may result from inhibited cell migration by the cross-links formed between collagen and extracellular macromolecules during the treatments, since it was not visible after de-epithelialization alone.[22] Interestingly, our observation appears to be supported by a long-term observation by Mastropasqua et al.[31,32] in patients who showed a reduction of cellular density 1 year after UVX treatment.

Myofibroblasts produce an opacity in the cornea due to diminished crystalline production[33] and the disorganized extracellular matrix that they synthetize and release. Those changes typically result in deep backscattering that typically appears 1 month after corneal surgeries involving stromal tissue removal, such as PRK.[34] However, several studies have shown that epithelial debridement without basement membrane disruption does not modify the corneal transparency.[22,35,36] In the current study we found that the haze decreased to minimal levels by 30 days post treatment, and we did not detect α-SMA-positive cells. These observations are consistent with those of other studies of UVX in which the number of α-SMA-positive cells was described as "scattered,"[9] "few,"[7] and "very few,"[21] suggesting that UVX and RGX cause little damage to the basal membrane.

In summary, our results indicate that the differences in localization and photoactivation of RF and RB in the cornea correlate with the deeper damage and slower kinetics of repair after UVX treatment than after RGX treatment. The covalent cross-links formed by both techniques appear sufficiently strong to produce a long-lasting increase in cornea stiffness despite the new cell population and remodeling of extracellular matrix.[23] The lesser extent of stromal damage produced by RGX treatment and the localization of increased stiffness closer to the anterior stromal surface suggest that RGX may be an effective treatment for keratoconus in thin corneas and for other conditions.

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