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Cornea & External Disease

The Efficacy and Safety of Oxygen-Enriched Rose Bengal and Green Light Corneal Crosslinking

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Purpose: To evaluate the efficacy and safety of Rose Bengal (RB) and green light crosslinking (RGX) with supplemental oxygen.

Methods: Rabbits were divided into three groups. Rabbits in the control group were de-epithelialized and served as controls. Rabbits in the RGX-nO_2 group were de-epithelialized, stained with 0.1% RB for 20 minutes, and then exposed to green light at 0.25 W/cm^2 for 10 minutes in an ambient atmospheric oxygen environment. Rabbits in the RGX-hO_2 group received the same treatment as group 2, but supplemental oxygen was given to the ocular surface during irradiation. Central corneal thickness was measured at various points during RGX. After RGX, corneal microstructure, corneal biomechanics, and resistance to enzyme lysis were evaluated.

Results: RGX-hO_2 and RGX-nO_2 significantly increased the corneal tensile strength by factors of 1.76 and 1.46, respectively. The times for complete corneal digestion within the RGX-hO_2, RGX-nO_2, and control groups were 23.2 $\pm$ 1.1 hours, 18.0 $\pm$ 2.0 hours, and 8.4$\pm$ 0.9 hours. The average collagen fibril diameters (43.58 $\pm$ 0.93 nm, 40.46 $\pm$ 1.11 nm, and 35.25 $\pm$ 0.75 nm, respectively) and surface-to-surface spacing (11.67 $\pm$ 1.91 nm, 15.51 $\pm$ 1.66 nm, and 24.93 $\pm$ 1.95 nm, respectively) also differed statistically among the three groups. No evidence of endothelial or stromal cell damage was detected by endothelial staining or TUNEL assay.

Conclusions: Increasing the concentration of oxygen during the irradiation process enhances RGX efficacy without causing obvious damage to a rabbit's endothelium or stromal cells.

Translational Relevance: The study can potentially help with improvement of the existing clinical crosslinking protocol.

Introduction

Keratoconus is the most common ectatic corneal disease and is characterized by progressive corneal thinning and steepening, resulting in increasing irregular astigmatism and myopia.[1] Corneal crosslinking (CXL) is a treatment that creates covalent bonds between collagen molecules and halts the progression of keratoconus.[1] Riboflavin and ultraviolet A (UVA) light crosslinking (UVX) has for some years been successfully applied in clinical practice, and the Dresden protocol in particular is considered the standard for CXL because of its excellent efficacy. However, the Dresden protocol has some drawbacks, including its time consumption, endothelium toxicity,[2–4] and the general requirement that corneal thickness must be limited to 400 µm so as to not exceed the endothelial cell toxicity threshold.[5] Rose Bengal (RB) and green light crosslinking (RGX) is another CXL technique that excites a photosensitizer (in this case, RB) with green light to produce photochemical crosslinking. Because RB tightly binds to tissue collagen and has limited penetration depth into the corneal stroma, RGX does not damage the corneal endothelium; thus, it is a promising candidate for patients with thin corneas.[6,7] Furthermore, RGX significantly increases corneal stiffness with more rapid treatment (12 to 30 minutes in total) than the Dresden protocol.

RGX acts by both singlet oxygen ($^1O_2$; oxygen-dependent, type I) and electron transfer (oxygen-independent, type II) pathways.[8] Although both pathways generate crosslinking, the oxygen-dependent pathway generates reactive oxygen species (ROS) more efficiently than the type II pathway. Oxygen is a key factor affecting the efficacy of CXL. Corneas irradiated in a hyperoxic environment were found to be stiffer than those irradiated in an O_2-free environment; thus, lack of oxygen significantly weakens the corneal mechanical effect of RGX in rabbit eyes.[8]

The aim of this study was to verify whether supplemental oxygen can increase RGX efficacy in vivo. A high concentration of oxygen was provided to the ocular surface continuously during irradiation. After RGX, the corneas were evaluated by stress–strain testing, measuring resistance to collagenase digestion, and carrying out transmission electron microscopy (TEM). Because the reaction with RB can induce ROS, it may also pose a potential risk to the endothelium. It is necessary to explore whether the increase of ROS with the supply of oxygen will cause damage to the corneal endothelium; therefore, the safety of RGX for the corneal endothelium was assessed with endothelial staining and TUNEL assay.

Materials and Methods

Animals

Thirty male New Zealand rabbits (3–6 months old, weighing 2.0–2.5 kg) were provided by the experimental animal center of Wenzhou Medical University. All of the rabbits were ordinary grade, without eye disease. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and strictly observed the requirements of animal welfare.

Reagents

The 0.1% weight/volume RB solution was prepared by dissolving RB powder (Sigma Aldrich, St. Louis, MO) in phosphate buffered saline (PBS; Solarbio, Beijing, China). The 0.2% weight/volume type II collagenase solution was prepared by dissolving collagenase powder (Worthington Biochemical, Lakewood, NJ) in PBS and was stored at 4°C.

RGX Procedure

Thirty-three rabbits were randomly divided into three groups (11 rabbits per group). The RGX procedure was performed after general anesthesia by intramuscular injection of sumianxin II and Zoletil into the thigh muscles and ocular surface anesthesia of the rabbit eyes with promecaine hydrochloride eye drops. As shown in Figure 1, after general and ocular anesthesia of the rabbit, the central 8-mm-diameter corneal epithelium was removed with a corneal scraper. Rabbits in group 1 served as controls. Rabbits in group 2 (RGX-nO_2 group) received RGX under normal oxygen conditions, and rabbits in group 3 (RGX-hO_2 group) received RGX under the condition of high oxygen concentration.

In the RGX-nO_2 group, 0.1% RB solution was applied to the cornea for 20 minutes using a well, followed by the irradiation (0.25 W/cm^2 for 10 minutes to deliver doses of 150 J/cm^2) with 532-nm green light on the cornea. The irradiation was performed in air with a normal oxygen concentration. The RGX-hO_2 group was subjected to the same treatment, except for irradiation in a hyperoxia environment with oxygen (95%, 5 L/min) provided on the cornea surface constantly by a Haier medical oxygen concentrator (Shanghai Haier Medical Technology, Co., Shanghai, China). Additional 0.1% RB solution was added to the cornea after 3.3 and 6.6 minutes of irradiation in both RGX groups. The central corneal thickness

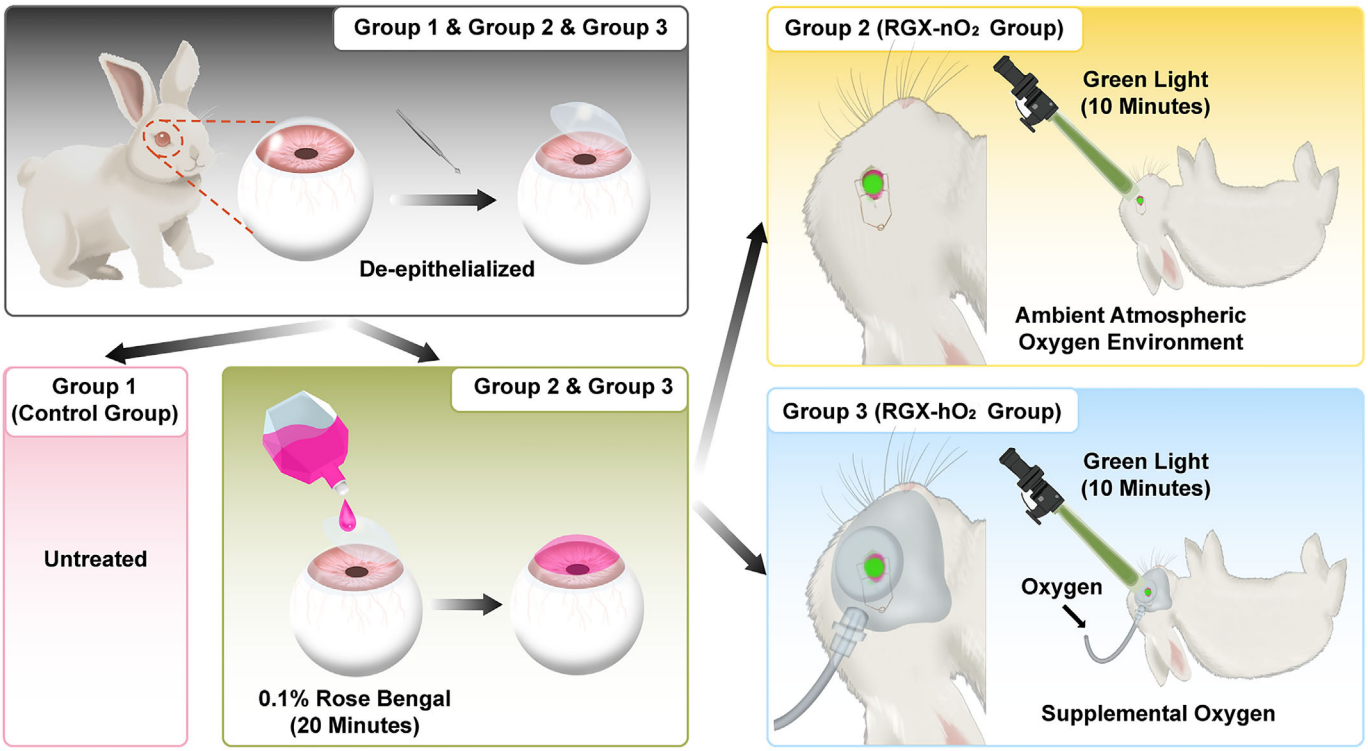

Figure 1. Animal grouping and treatment.

(CCT) before de-epithelization, after RB infiltration and after green light irradiation ($n = 8$ eyes per group), was measured by an A-type ultrasound pachymetry (SP-3000; Topcon Corporation, Tokyo, Japan). The rabbits were euthanized instantly by inhalation of excessive amounts of carbon dioxide under complete general anesthesia after RGX. After confirming complete animal death, corneas were removed for the follow-up experiments.

Stress–Strain Test ($n = 6$ Eyes Per Group)

Immediately after euthanasia of the rabbits, 3-mm × 10-mm corneal strips with 3-mm sclera remnants at both ends were taken from the middle of the cornea along the inferior–superior direction. Each strip was then mounted vertically between the two clamps of the testing machine (3343; Instron, Norwood, MA). After three loading/unloading cycles, the load was gradually increased at a rate of 2 mm/min until the strip was stretched to 20% deformation and stress–strain curves could be obtained. The Young's modulus values at 2%, 4%, 6%, 8%, and 10% deformation were numerically equal to the slope of the curve at these corresponding positions.

Enzymatic Digestion ($n = 5$ Eyes Per Group)

After the euthanasia of the rabbits, the central 8-mm-diameter cornea discs were immediately trephined and immersed into a plate containing 0.2% type II collagenase, then placed in a constant temperature shaker (THZ-100, Shanghai Yiheng Scientific Instrument Co., Shanghai, China) at 37°C. During the digestion process, the remaining corneal tissues were photographed at regular intervals until completely digested. The area of undigested cornea was measured by ImageJ 1.51j8 (National Institutes of Health, Bethesda, MD), and corneal area–digestion time curves were drawn.

Transmission Electron Microscopy ($n = 6$ Eyes Per Group)

After the euthanasia of the rabbits, the central 1 mm × 1.5 mm of the cornea was cut out and immediately fixed in 2.5% glutaraldehyde solution overnight at 4°C. The tissues were washed three times for 10 minutes each in PBS followed by post-fixation in 1% osmic acid for 1 hour. They were thoroughly washed in PBS and deionized water and immersed in 1% uranium acetate to be stained for 1 to 2 hours. After uranium acetate

staining, the tissues were dehydrated in an incremental series of acetone concentrations and embedded with the acetone and embedding solution (Araldite Epon-812; Huntsman Advanced Materials, The Woodlands, TX). Finally, the tissue was immersed in pure embedding agent and placed in an oven at 45°C for 3 hours and 65°C for a further 48 hours. Ultrathin sections of the anterior corneal stroma were cut from the tissue blocks and observed using a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan). ImageJ 1.51j8 was used to analysis the minimum diameter of collagen fibers (CFs), as well as the surface-to-surface spacing and density of CFs, referring to the method of Zheng et al.[9] After choosing the area of the image, the number and total area of the fibrils were measured. Incomplete fibrils situated at the edge of the region were not counted. Then, the average fibril diameter and average surface-to-surface spacing were calculated, based on the fibrils being circular in cross-section.

Endothelium Staining ($n = 2$ Eyes Per Group)

We used 0.2% trypan blue (Sigma-Aldrich) and 0.2% alizarin red (pH 4.7; Sigma Aldrich) to stain the nucleus and cytoskeleton of the corneal endothelium. The corneal endothelial cells were photographed with an upright microscope (DM750; Leica Microsystems, Wetzlar, Germany).

TUNEL Assay ($n = 3$ Eyes Per Group)

The frozen corneal sections were stained with a TUNEL Apoptosis Test Kit (Roche, Basel, Switzerland) and subsequently stained with 4and subsequently stained wit (DAPI) before being observed under a confocal microscope (LSM 710; Carl Zeiss Microscopy, Oberkochen, Germany), with 488-nm excitation light for TUNEL and 405-nm excitation light for DAPI. DNase was added to both eyes of the last rabbit as a positive control.

Statistical Analysis

Chart data were analyzed using Prism 8.4 (GraphPad, Boston, MA). Quantitative results, including CCT, Young's modulus, dissolution times, and CF diameter and distance, are expressed as the mean ceSD. Differences between the RGX groups and the control group were analyzed using independent samples *t*-tests. Comparisons among all the three groups were analyzed using one-way analysis of variance (ANOVA). $P < 0.05$ indicated statistical significance.

Results

Stress–Strain Test

The stress–strain curves are shown in Figure 2. At 10% strain, the Young's modulus values for the RGX-hO_2 group (33.66 ± 3.40 MPa; $P < 0.0001$) and the RGX-nO_2 group (27.86 ± 2.14 MPa; $P = 0.001$) were both significantly larger than in the control group (19.13 ± 3.36 MPa). The difference between the two RGX groups was statistically significant ($P = 0.0214$) (Table 1).

Enzymatic Digestion

The dissolution times for the RGX-hO_2 group (23.2 ± 1.1 hours) and RGX-nO_2 group (18.0 ± 2.0 hours) were both significantly longer than for the control group (8.4 ± 0.9 hours; both $P < 0.0001$). The RGX-hO_2 group was digested more slowly than the RGX-nO_2 group ($P = 0.0002$) (Fig. 3).

Transmission Electron Microscopy

In transverse sections, the CFs of the three groups maintained their original shape, and no morphological damage was observed (Figs. 4A–4C). The CF diameters of the RGX-hO_2 group (43.58 ± 0.93 nm; $P < 0.0001$) and the RGX-nO_2 group (40.46 ± 1.11 nm; $P < 0.0001$) were both greater than for the control group (35.23 ± 0.82 nm). The surface-to-surface spacing of the CFs in the RGX-hO_2 group (11.67 ± 1.91 nm; $P < 0.0001$) and the RGX-nO_2 group (15.51 ± 1.66 nm; $P < 0.0001$) were both significantly reduced

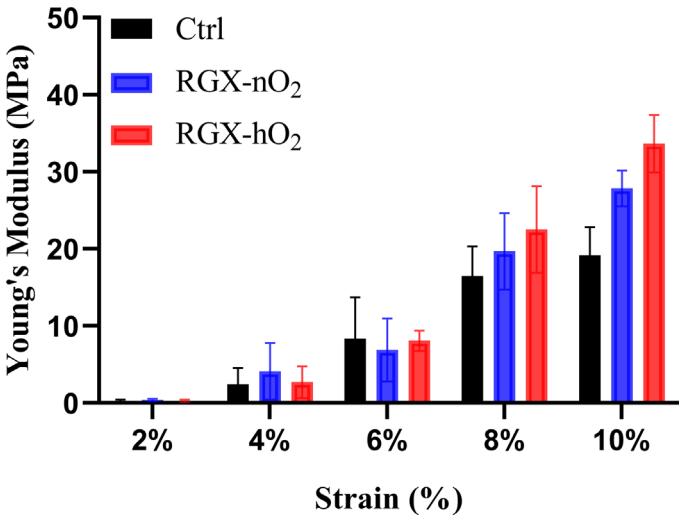

Figure 2. Young's modulus of corneal strips for different strains ($n = 6$).

Table 1. Young's Modulus at Different Strains ($n = 6$)

	Control	RGX-nO_2	RGX-hO_2
Young's modulus at 10% (MPa)	19.13 ± 3.36	27.86 ± 2.14	33.66 ± 3.40
Young's modulus at 8% (MPa)	16.48 ± 3.52	19.68 ± 4.51	22.52 ± 5.12
Young's modulus at 6% (MPa)	8.35 ± 4.92	6.89 ± 3.72	8.08 ± 4.92

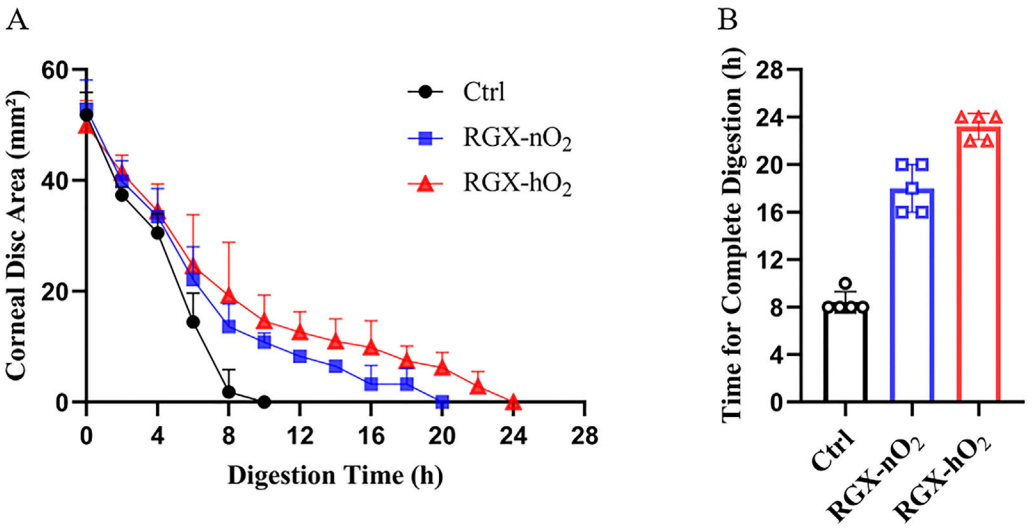

Figure 3. (**A**) Curves of the residual corneal disc area in collagenase. (**B**) Histogram of the time for complete digestion ($n = 5$).

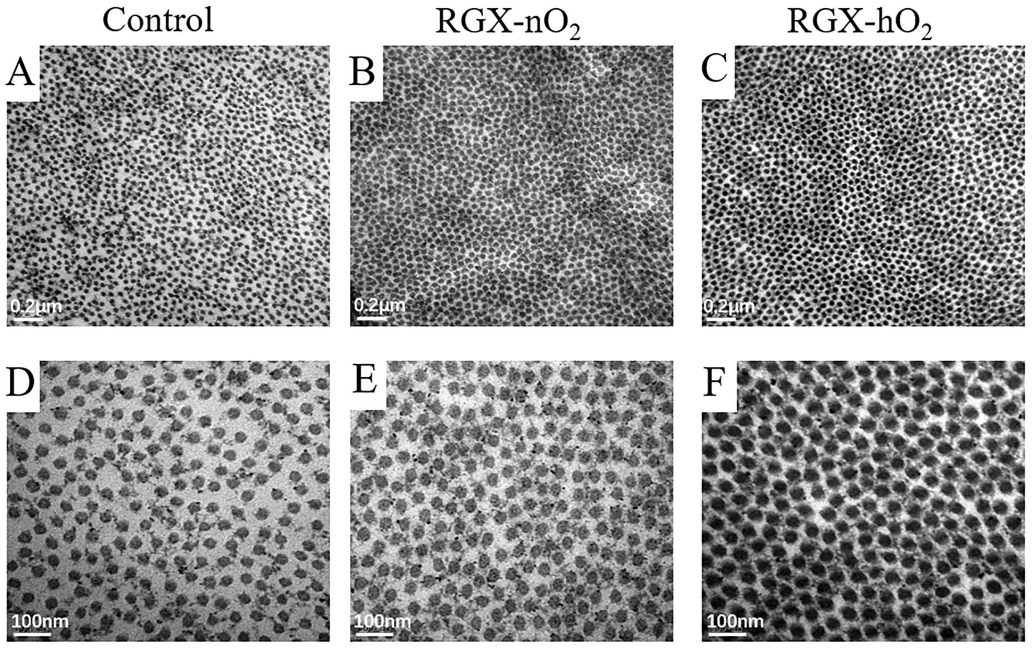

Figure 4. Transmission electron microscopy of the corneal stroma ($n = 6$). *Scale bars*: 0.2 µm (**A**–**C**), 100 nm (**D**–**F**).

compared to the control group (24.92 ± 2.13 nm) (Figs. 4D–4F, Fig. 5B). The differences in CF diameter ($P = 0.0003$) and spacing ($P = 0.0129$) between the two RGX groups were significant (Fig. 5A). Although the collagen fiber density of the RGX-hO_2 group was slightly higher than in the other two groups, there was no statistical difference among the three groups ($P > 0.05$) (Fig. 5C).

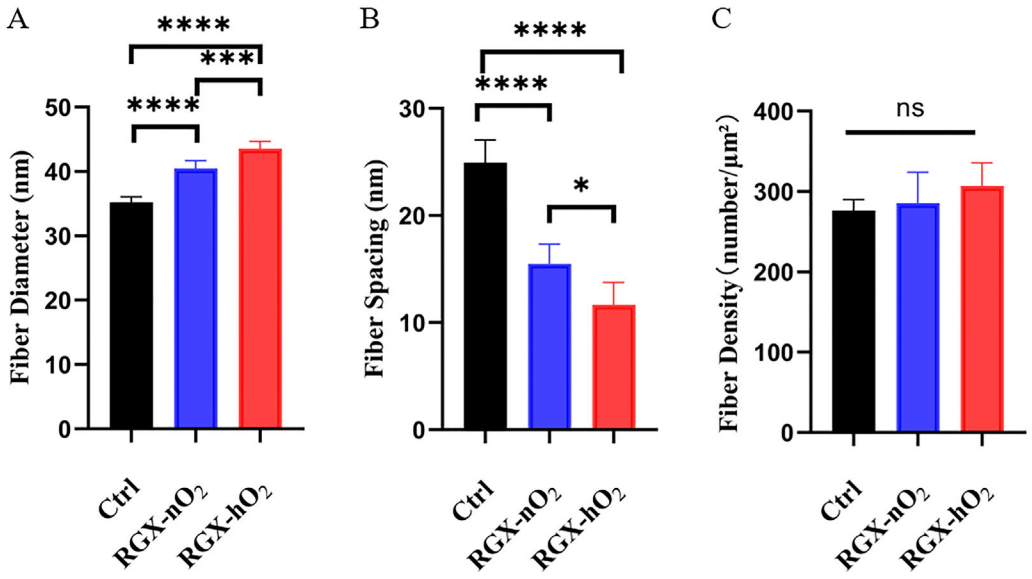

Figure 5. The minimum diameter, surface-to-surface spacing, and density of CFs ($n = 6$).

Table 2. Corneal Central Thickness During Treatment ($n = 8$)

	RGX-nO_2	RGX-hO_2	*P*
CCT before de-epithelization (μm)	356.1 ± 28.7	359.4 ± 24.7	0.9967
CCT after RB infiltration (μm)	422.0 ± 23.1	428.6 ± 30.4	0.9732
CCT after irradiation (μm)	303.6 ± 17.3	254.0 ± 62.0	0.0177

Corneal Central Thickness

The CCT values at each time point are listed in Table 2. Before de-epithelialization, the CCT of both RGX groups was around 355 μm, without a statistically significant difference. After RB infiltration, the CCT of both groups increased by about 65 μm. After irradiation, the CCT significantly decreased, whereas the RGX-hO_2 group showed a statistically greater decrease.

Endothelium Staining

The morphology of endothelial cells showed no significant difference between the two RGX groups and the control group, as the endothelial structures were intact (Figs. 6A–6C). Due to the red background caused by RB, the image contrast of the two RGX groups was much lower than for the control group, but the boundaries and morphology of the endothelial cells were presented.

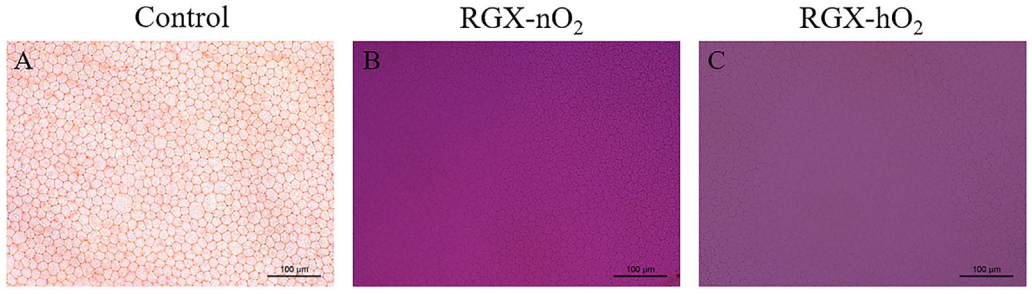

Figure 6. Endothelial cell staining images after RGX surgery. *Scale bars*: 100 nm.

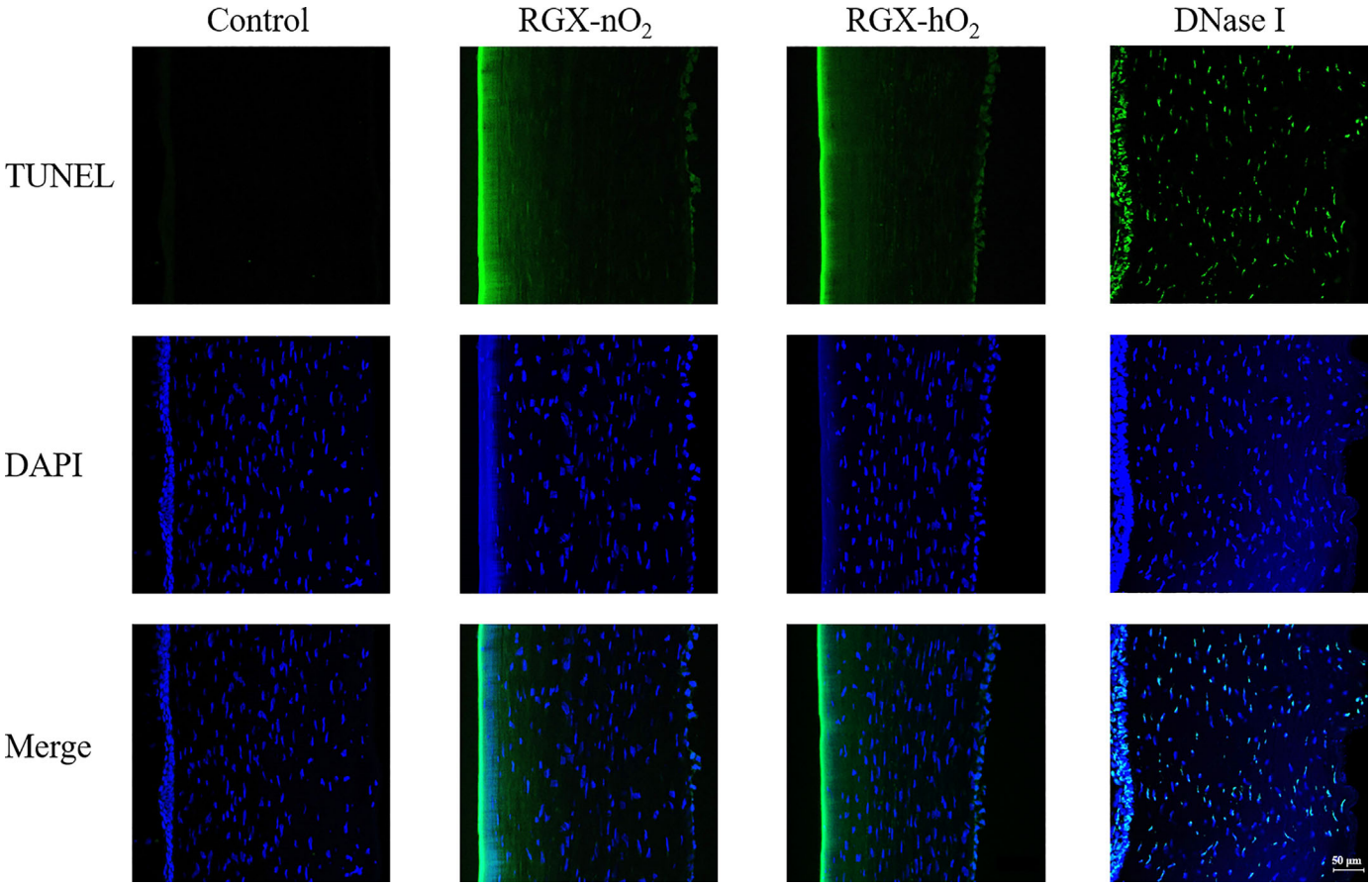

Figure 7. Cell apoptosis labeled with TUNEL assay and cell density labeled with DAPI. *Scale bars*: 50 µm.

TUNEL Assay

The corneal section micrographs showed no apoptotic cells in the two RGX groups or in the control group. The green fluorescence of the two RGX groups did not exhibit cellular morphology and was caused by RB rather than apoptosis. Apoptosis was observed in corneal epithelial cells, stromal cells, and endothelial cells in the DNase I group (Fig. 7).

Discussion

CXL is a photochemical reaction between the incident light and the photosensitizer in the corneal stroma. When irradiated by green light, RB is excited and generates ROS, and the ROS further react with various molecules within the tissue to induce crosslinking. The oxygen content on the corneal surface is an important factor affecting CXL protocols.

The oxygen-dependent pathway in the UVX protocol has been recognized. Corneas treated under an oxygen concentration of 21% showed a significant Young's modulus increase compared to those treated under oxygen concentrations less than 0.5%, and the Young's modulus of the latter was similar to that of untreated controls.[10] Increasing oxygen concentration can improve the efficacy of UVX, especially for transepithelial corneal crosslinking with a high intensity of irradiation.[11] Seiler et al.[12] used probes to measure oxygen as a function of depth during CXL. They found that oxygen levels dropped with corneal depth, but oxygen supply in the deeper layers was considerably increased within a hyperoxic environment. Furthermore, previous studies have shown that a hypoxic environment noticeably weakened the biomechanical effect of both UVX and RGX.[8,10]

Hill et al.[13] found that the stromal oxygen concentration was increased about fivefold after the environmental oxygen concentration was enriched from 20% to 90% during accelerated UVX (30 mW/cm^2). Both the experimental results and the CXL mechanism supported that oxygen enrichment may prevent oxygen depletion and thus enhance CXL efficiency.[14] Thus, most data indicate that intrastromal oxygen diffusion and oxygen consumption caused by the photochemical reaction are factors that limit CXL efficiency.

Diakonis et al.[15] supplied an external O_2 gas stream (4 L/min pure O_2) on the surface of the cornea during accelerated 30-mW/cm^2 UVX treatment, but in this case it failed to produce stiffer corneas. They attributed the results to the 3-minute irradiation time used in the protocol, which is too short for oxygen diffusion; however, the RGX technique requires a 10-minute irradiation time, which may allow oxygen to diffuse and thereby facilitate the photochemical reaction.

There is no research exploring the effect of oxygen supply on RGX so far. Comparing RGX and UVX, there are significant differences in irradiance, as well as the penetration depth of photosensitizers into the cornea. Krueger et al.[16] found that the oxygen consumption rate was associated linearly with the UVA irradiance, being about 5 times faster with 16-mW/cm^2 than 3-mW/cm^2 irradiance. The intensity of green light is 0.25 W/cm^2, much higher than UVA. Therefore, we speculated that there is also a difference in the consumption of oxygen between the two crosslinking methods, and RGX may require more oxygen than UVX. We further speculated that increasing the oxygen supply on the corneal surface would increase the efficacy of RGX. The ambient oxygen environment (~20% oxygen concentration) maintained an oxygen concentration of 13% at 230-µm stromal depth.[13] As shown by Thaware et al., 90% to 99% oxygen concentrations were achieved at the 1-L/min oxygen flow rate.[14] Our previous studies have shown that the infiltration depth of RB in the cornea stroma is approximately 200 µm after 20 minutes of infiltration. The flow rate of 5 L/min used in the current study should therefore have been sufficient over the depth at which crosslinking occurred.

Verter et al.[17] performed RGX (100 J/cm^2) to enhance the attachment of amnion, which is used in some corneal surgeries, to rabbit cornea. The intraocular pressures that broke the seal produced by RGX were 178 ± 12 and 208 ± 81 mm Hg, respectively, in air and oxygen, whereas the pressure was only 66 ± 44 mm Hg in nitrogen, which was not different from the control group, clearly indicating that oxygen was involved in the reaction. This study suggests that oxygen can enhance the crosslinking between the amnion and the cornea, but it does not shed light on whether oxygen can increase crosslinking inside the corneal tissue.

The results of our study indicate that sufficient oxygen in the corneal stroma can increase RGX efficacy. From our experimental results, at 10% strain, the Young's modulus values for the RGX-hO_2 and RGX-nO_2 groups were increased, respectively, 1.76-fold and 1.46-fold compared with the control group. The digestion time in the RGX-hO_2 group was 1.29 times greater than in the RGX-nO_2 group, and the 10% Young's modulus was 1.21 times greater. In other words, RGX conducted in a high oxygen environment showed better corneal biomechanical properties and higher anti-collagenase digestion ability. Moreover, the CFs crosslinked under high oxygen concentrations had the largest diameter and the smallest spacing, indicating that the corneal CFs in the anterior stroma became thicker and more tightly packed after RGX; this may contribute to the improved biomechanical properties. The diameter of CFs in our control group was similar to that reported by Wollensak et al.,[18] who found that in the anterior stroma the CFs diameter in the UVX corneas was increased by 12.2% (3.96 nm) compared with the control group. The CFs diameters in the RGX-hO_2 and RGX-nO_2 groups in our study were increased by 23.7% (8.35 nm) and 14.8% (5.23 nm), respectively, illustrating that RGX-hO_2 may thicken the corneal fibers even more than UVX. Wollensak et al.[18] suggested that the possible reason for the increase in diameter that they observed was that crosslinking in collagen pushes collagen molecules apart, leading to an increase in intermolecular spacing of CFs, thereby causing a diameter increase. Similarly, Faulborn et al.[19] used electron microscopy to observe autopsied eyes from patients with long-standing diabetes and found that the CFs in central vitreous were enlarged and aggregated.

As shown by the endothelial staining results, RGX-nO_2 caused no serious damage to the endothelium, which is consistent with many previous studies.[7,18,20] The CCT after RGX-hO_2 was about 50 µm thinner than in the RGX-nO_2 group (303.6 ± 17.3 vs. 254.0 ± 62.0; P = 0.0177). This may be the result of evaporation of stromal water by airflow on the surface, which would be consistent with the increased fibril density and closer packing in the TEM results. Nonetheless, dehydration during irradiation may cause thinning, which threatens endothelial safety. The endothelial staining and TUNEL assay further confirmed that RGX with oxygen supplementation did not cause significant damage to the corneal endothelium. This may be attributed to fact that RB binds to collagen, absorbs green light, and protects endothelial cells, at least at this thickness of 254.0 ± 62.0 µm.

There are some shortcomings in this study. First, with respect to the maximum oxygen flow rate of 5 L/min provided by the oxygen generator, future trials are necessary to clarify the optimal oxygen concentration required under various RGX conditions such as RB concentration and green light irradiance. In addition, the intrastromal oxygen distribution would have to be measured with an oxygen-sensing microelec-

trode inserted at various stromal depths. The biomechanical properties at different depths of the cornea should also be explored. Moreover, the biomechanics and safety of the rabbit cornea after RGX require long-term follow-up, which is our future aim. The number of corneas evaluated is another limitation of the study, which may impact the robustness and generalizability of the findings. In summary, sufficient oxygen in the corneal stroma enhanced RGX efficacy without damaging endothelial cells.

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* RG, MC, and WX contributed equally as first authors.

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